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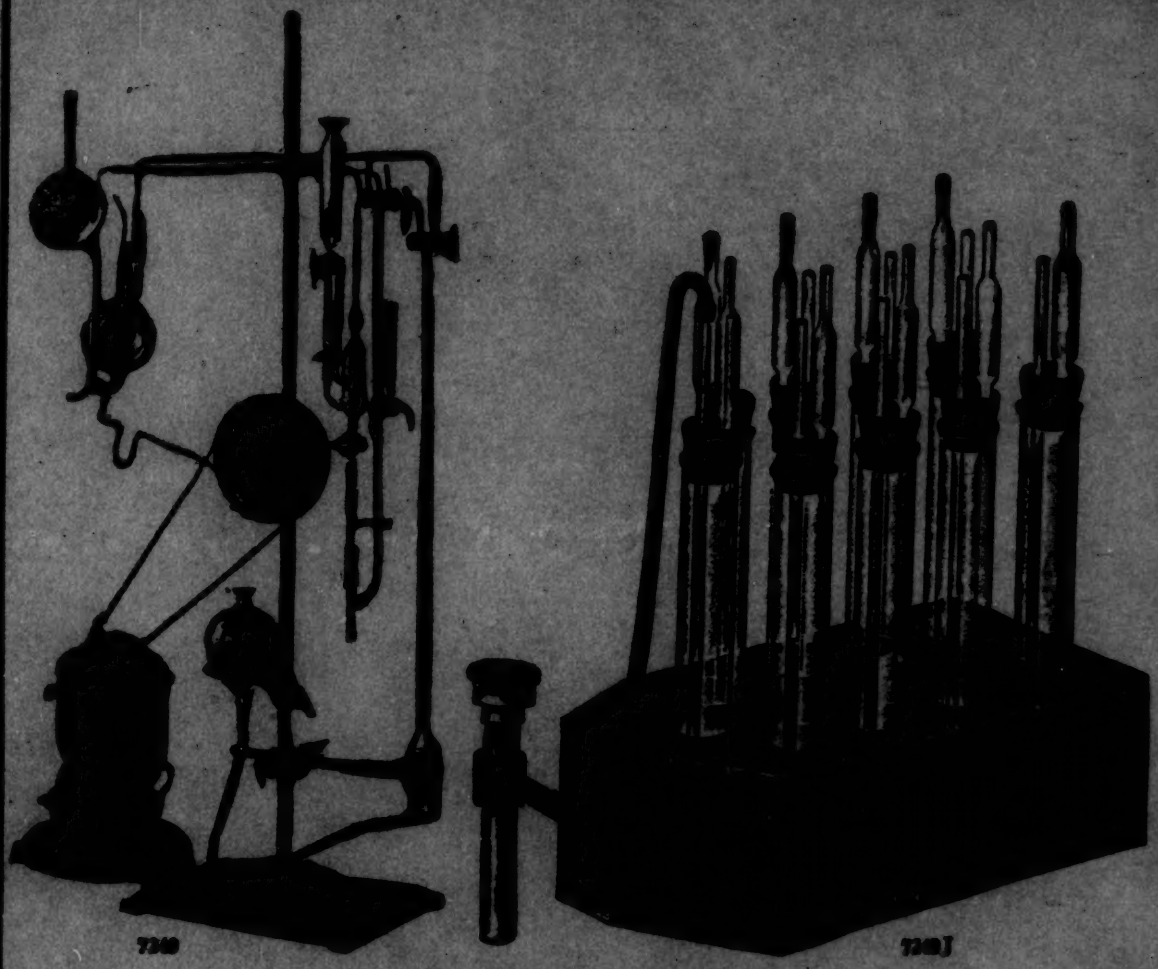

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THE AMINO-ACID MINIMUM FOR MAINTENANCE AND GROWTH, AS EXEMPLIFIED BY FURTHER EXPERIMENTS WITH LYSINE AND TRYPTOPHANE.[1]

By THOMAS B. OSBORNE and LAFAYETTE B. MENDEL.

With the Cooperation of Edna L. Ferry and Alfred J. Wakeman.

(*From the Laboratory of the Connecticut Agricultural Experiment Station and the Sheffield Laboratory of Physiological Chemistry in Yale University, New Haven.*)

(Received for publication, March 18, 1916.)

The current view of the construction of new tissue involves the synthesis of new protein as an essential feature. Growth will be limited, therefore, by *any* factor which prevents this synthesis. The lack of any component amino-acid which cannot be manufactured directly in suitable amounts in the body represents such a limiting factor. This explains why adequate growth has never been obtained with rations in which the nitrogenous components fail to furnish sufficient proportions of certain amino-acids such as tryptophane, lysine, or cystine. The wear-and-tear features of the metabolism of maintenance without growth may also call for a certain quota of some, if not all, of these same amino-acids.

In an earlier paper we expressed the following conclusion: "Obviously the relative values of the different proteins in nutrition are based upon their content of those special amino-acids which cannot be synthesized in the animal body and which are indispensable for certain distinct, as yet not clearly defined processes which we express as maintenance or repair."[2] The well known "law of minimum" may be expected to apply here as in other nutrition phenomena. In *growth* some of the amino-acids *must* become an integral part of new protein in the body; so that their participation

[1] The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, xvii, 340.

in this process can be expressed in quantitative terms. It is by no means equally certain that a precisely similar rôle belongs to them in the metabolism of ordinary *maintenance;* for a few specific protein derivatives alone may here function in regulatory performances rather than as the direct source of tissue repair or construction. In an extreme view it might be assumed, for example, that the only reason for the destruction of any protein at all, in maintenance, would be to liberate a small amount of one or two amino-acids to engage in some hormone-like physiological duty upon which proper metabolism might depend.[3] In any event, if these newer views are tenable, individual indispensable amino-acids ought to bear some quantitative relation to the nutritive needs of an organism, so that the efficiency of a diet can be modified by varying the supply of any one of these amino-acids.

Willcock and Hopkins[4] have shown that when zein forms the sole protein of the diet the life of young mice was greatly prolonged by adding *tryptophane* to the diet. Their experiments have been confirmed and extended by many of our own with albino rats, and the results have already been published.[5] We have also shown that growth does not occur when *lysine* is lacking from the diet, but takes place promptly on adding this amino-acid to the otherwise adequate food.[6] Furthermore, addition of these amino-acids to diets which are quantitatively deficient in either one of them is

[3] The actual amount of dietary protein required for the maintenance metabolism is very small. It is possible to maintain rats on rations containing as little as 2.5 per cent of protein calories (see Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 351) and similar facts have been recorded for man. We have attempted to learn whether it would be possible to maintain rats on a non-protein diet with additions of tryptophane alone, or together with cystine, histidine, tyrosine, phenylalanine, proline, and ammonium citrate, or urea. All such attempts failed, even when the supply of energy in the form of non-protein substances was liberal and the food contained all of the necessary inorganic salts and "food accessories," and in addition at least 0.5 per cent of protein, present in the "protein-free" milk. On such diets the rats declined just as rapidly as when the amino-acid additions were not made.

[4] Willcock, E. G., and Hopkins, F. G., *J. Physiol.*, 1906, xxxv, 88.

[5] Osborne and Mendel, *Science*, 1913, xxxvii, 185; *J. Biol. Chem.*, 1914, xvii, 325; 1914, xviii, 1; 1915, xx, 351.

[6] Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 325 (see especially Chart IV, p. 345); 1915, xx, 351.

promptly followed by an increased rate of growth.[7] We have also presented data showing a greater rate of growth after adding *cystine* to diets containing casein as the sole protein, for casein is presumably quantitatively deficient in cystine.[8]

In the present paper we furnish data which show that the need for tryptophane and lysine is governed by the "law of minimum," the rate of growth increasing with increasing amounts of these amino-acids furnished by the food until the normal rate is attained. When larger quantities of these amino-acids are supplied growth is not made more rapidly, for the construction of new tissue is no longer limited by deficiencies in the requisite supply of any element of the food, but by the natural capacity of the animal to grow.

Experience has shown that a certain minimal amount of tryptophane is essential for maintenance without growth. That lysine is not needed to the same degree for maintenance is shown by an experiment already published, in which Rat 1892 was maintained at an almost constant body weight of 50 gm. for 182 days on a food containing zein as its sole protein with an addition of tryptophane equal to 3 per cent of the zein.[9] Although no lysine was available, other than that which the rat might obtain from its own musculature, which wasted away during the 6 months of the experiment, and from the small amount of protein in the "protein-free" milk, which at the most could furnish a quantity not greater than 0.13 per cent of the food, or 0.7 per cent of the zein, the animal remained vigorous and at once began to grow when lysine equal to 3 per cent of the zein was added to the diet.

[7] Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 325. (See Charts III, p. 344, and VIII, p. 349.)

[8] Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 351. (See Chart IV, p. 373.)

[9] Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 351. (See Chart IX, p. 378.) The composition of the food mixture was:

	gm.
Zein	17.46
Tryptophane	0.54
"Protein-free" milk	28.00
Starch	27.00
Butter fat	18.00
Lard	9.00
Water	15.00

Whether maintenance is possible when lysine is completely lacking from the diet has never been demonstrated, owing to the unavoidable presence of a little protein in the "protein-free" milk, but, in any event, the need for lysine is not comparable with that for tryptophane, for with the similar small amounts of this amino-acid available in the muscles of the animal and also in the protein of the "protein-free" milk, the animals decline rapidly on the zein foods unless tryptophane is added in sufficient quantity, which for long periods we have found to be equal to 3 per cent of the zein. Even additions of lysine to the zein foods do not prevent the decline unless tryptophane is also added.[10] Another record of this sort is shown in Chart I for Rat 1905.[11]

In the experiment just cited a unique evidence of the inhibition of growth was presented throughout the whole period of 6 months during which the rat remained at constant body weight. A small patch of hair on the animal's back had been dyed red for purposes of identification before the experiment was begun. This remained unchanged until lysine was added to the diet at the end of 182 days, and growth was resumed, when the color soon disappeared. New growth had become possible in the hairs as in other parts of the body.

The record of the experiment just referred to, for Rat 1892, is given in Chart I. In the records of two rats, 2475 and 2476, presented in Chart II, it is shown that zein food can be made adequate for maintenance and even for slight growth by additions of both lysine and tryptophane with far smaller proportionate quantities of the latter than was the case where tryptophane alone was added. We are unprepared to explain the exact significance of this conserving effect of the presence of lysine. The experiments were of comparatively short duration, having been planned to show the influence of different proportions of these two amino-acids. In Chart III are recorded results of experiments to de-

[10] Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 325. (See Rat 1900♂, Chart VI, p. 347.)

[11] The sudden rise in body weight indicated in the chart just prior to the death of this animal was due to a pathological collection of fluid in the abdominal cavity. It must not be interpreted as an indication of the ability of the added lysine to render the zein food adequate for maintenance.

termine the quantitative limits for the proportions of lysine and tryptophane needed to promote good growth with zein foods. (See also Rats 2154 and 2161, Chart I.) These graphic representations of the body weight show strikingly the effect of increasing or decreasing the quantities of lysine and tryptophane. Both must be present in sufficient amount before maximal effects can be obtained. Furthermore, we have already intimated that for ideal growth zein ought perhaps also to be supplemented by additions of arginine and histidine which it yields in rather small proportions.[12]

The quantitative rôle of lysine is shown even more strikingly in the case of gliadin foods. When gliadin is the only protein in the ration, growth is very slow, if it occurs at all.[13] Gliadin yields tryptophane; and its lysine content is now estimated at about 0.92 per cent.[14] The adequacy of the gliadin foods for the maintenance of rats (without noteworthy growth) at various ages represented by correspondingly different body weights has been demonstrated in earlier papers. In these experiments the foods contained 18 per cent of gliadin. We have since found that foods containing only 9 per cent of gliadin can maintain the body weight of rats at least for short periods. With foods containing only 2 per cent of gliadin maintenance is no longer possible.[15] Gliadin can supplement zein food, so as to make the latter adequate for maintenance.[16] Inasmuch as isolated tryptophane acts in the same way, biological evidence for the presence of a considerable proportion of tryptophane in gliadin is thus afforded.

It has been shown that the addition of lysine equivalent to 3 per cent of the gliadin fed readily converts the gliadin food into a

[12] Osborne and Mendel, *J. Biol. Chem.*, 1914, xviii, 1. (See Chart I, p. 11.)

[13] Osborne and Mendel, *Carnegie Institution of Washington, Publication 156*, pt. ii, 1911; *Z. physiol. Chem.*, 1912, lxxx, 328; *J. Biol. Chem.*, 1912, xii, 473; 1912–13, xiii, 233; *Science*, 1913, xxxvii, 185; *J. Biol. Chem.*, 1914, xvii, 325.

[14] Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, 1915, xxii, 259.

[15] Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 351. (See Rats 2435 and 2463, Chart VIII, p. 377.)

[16] Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 325. (See Rat 1113 ♀, Chart I, p. 342.)

ration suitable for growth.[17] More recent attempts to ascertain the lower limit of this lysine requirement are presented in Chart IV (Rats 2054, 3 per cent lysine; 2268, 2271, 2 per cent lysine; 2363, 2366, 1.5 per cent lysine; 2294, 2291, 1 per cent lysine). A distinct effect upon the rate of growth is not apparent until this lowest addendum of lysine is reached. Recalling that the gliadin itself furnishes nearly 1 per cent of lysine, it is obvious that, to produce satisfactory growth, lysine equal to somewhat over 2 per cent of the protein must be present in the food. For the purposes of comparison the food intake and gains in body weight for a period of 3 weeks of rats of comparable size, some of which are referred to in Chart IV, are summarized below:

Lysine added to gliadin food.		Rat.	Initial body weight.	Food intake in 21 days.	Gain in body weight in 21 days.
As per cent of gliadin.	In 100 gm. food.				
per cent	*gm.*		*gm.*	*gm.*	*gm.*
3	0.54	1844 ♀ *	59.0	117.8	37.5
		1846 ♀ *	54.0	108.4	30.5
		1850 ♂ *	75.0	142.3	45.5
		2054 ♂ †	60.0	136.9	39.0
2	0.36	2268 ♂ †	69.0	150.2	35.0
		2271 ♂ †.	65.5	148.7	35.5
1.5	0.27	2363 ♂ †	66.0	136.7	30.5
		2366 ♂ †	67.0	130.2	25.5
1	0.18	2291 ♂ †	64.5	136.6	21.5
		2294 ♂ †	61.5	113.7	9.5
None.	0.00	240 ♀ ‡	65.5	110.8	3.0
		249 ♀ §	65.5	87.4	6.0
		254 ♀ **	66.5	95.3	8.0
		1850 ♂ *	65.5	103.1	8.0

* See Chart I, *J. Biol. Chem.*, 1914, xvii, 342.
† See Appendix, Chart IV.
‡ See Chart VII, *J. Biol. Chem.*, 1912, xii, 496.
§ See Chart VIII, *J. Biol. Chem.*, 1912, xii, 498.
** See Chart IX, *J. Biol. Chem.*, 1912, xii, 499.

[17] Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 325. (See Chart I, p. 342.)

Inasmuch as gliadin, which yields nearly 1 per cent of lysine, in addition to tryptophane, fails to promote growth until a supplement of 1 or more per cent of lysine is added to the food containing it, one can now readily understand why the zein food failed to promote growth satisfactorily, even with the addition of plenty of tryptophane, unless 2 per cent or more of lysine was furnished with the protein (see Charts II and III). Some growth may occur with smaller additions, but it is below the normal average and is limited by the proportion of the essential amino-acid present in smallest amount.

The application of some of these ideas regarding the indispensability of an adequate supply of certain amino-acids for growth has recently been attempted by Buckner, Nollau, and Kastle in the case of young chicks.[18] Their plan was to select mixtures of feeds which should be high and low, respectively, in lysine. The amount of this amino-acid was estimated by hydrolyzing the grain rations as a whole and determining the partition of nitrogen by the Van Slyke method. If the results of analyses made in this manner are trustworthy, the outcome of these feeding experiments is of much interest. They showed a very marked difference in the effects of the two grain mixtures, the rations of assumed low lysine content leading to decided retardation of growth. There are many variables besides that of the amino-acid yield in any attempt to compound food mixtures from ordinary feeds.

In a recent publication Abderhalden[19] has discussed what he designates as the "biologische Wertigkeit" of a number of the amino-acids obtained by the complete disintegration of protein with digestive enzymes. In several respects his results are comparable with studies which we have presented and repeatedly discussed in several communications in the past. His procedure has, in the main, consisted in feeding to dogs and rats mixtures of amino-acids obtained by digestion; and using the nitrogen balance as an evidence of nutritive equilibrium, he has ascertained the effect of such mixtures before and after the removal of individual amino-acids. In this way, quite in harmony with evidence which we have presented earlier, he has come to the conclusion that

[18] Buckner, G. D., Nollau, E. H., and Kastle, J. H., *Am. J. Physiol.*, 1915, xxxix, 162.

[19] Abderhalden, E., *Z. physiol. Chem.*, 1915, xcvi, 1.

tryptophane is an indispensable component of the dietary. Abderhalden's experiments with lysine, though pointing in the same direction, are far less conclusive.

SUMMARY.

Additional experiments are reported in this paper to show the part played by tryptophane and lysine in the metabolism of maintenance and growth. The quantity of these amino-acids available in the diet can be made the limiting factors which determine the nutritive equilibrium and possibilities for increment of size in an individual. They afford an important illustration of the "law of minimum" applied to essential nitrogenous components of the food supply.

APPENDIX.

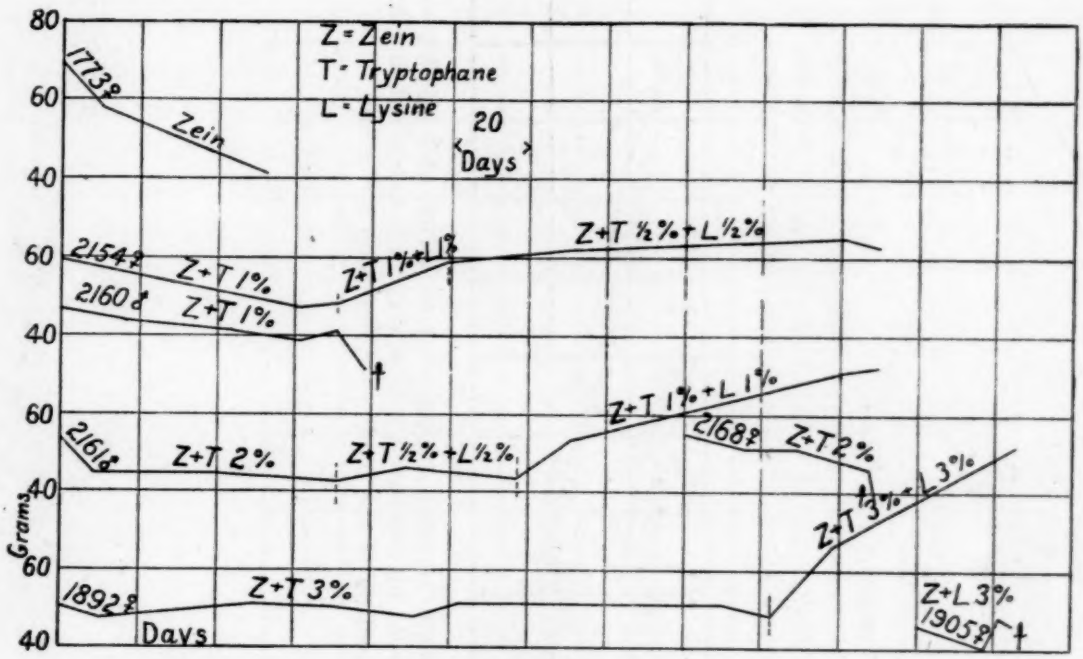

CHART I. Showing the effect of additions of varying proportions of tryptophane, lysine, or both, to zein foods. It will be noted that no increment of body weight takes place unless both of these amino-acids are added. The rate of growth appears to be limited by the proportions of these amino-acids furnished, thus illustrating the "law of minimum." Special attention is directed to the unique record of Rat 1892 described in detail in the text. The food, with which the lysine or tryptophane was incorporated, was composed essentially as follows:

	gm.
Zein + amino-acid	18
"Protein-free" milk	28
Starch	27
Butter fat	18
Lard	9
Water	15

In all cases the quantities of amino-acids used are expressed in percentages of the protein present—not in terms of the total food.

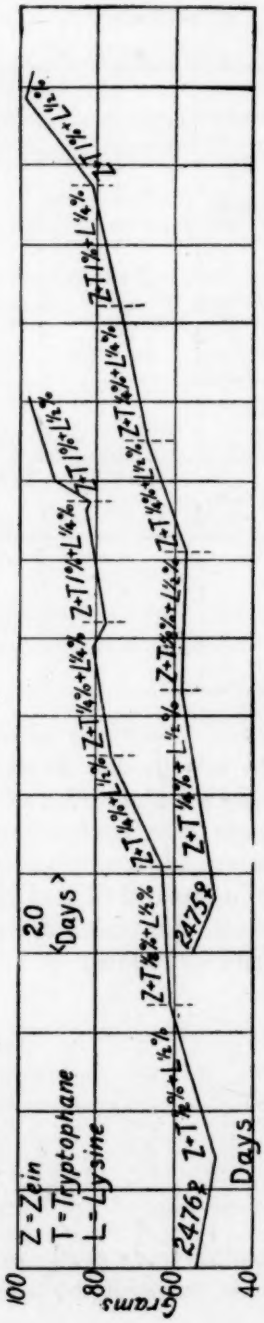

CHART II. Showing the influence of additions of small proportions of lysine and tryptophane upon the body weight of small rats. These should be compared with the records exhibited in Chart I, in connection with which the general composition of the foods used has been indicated.

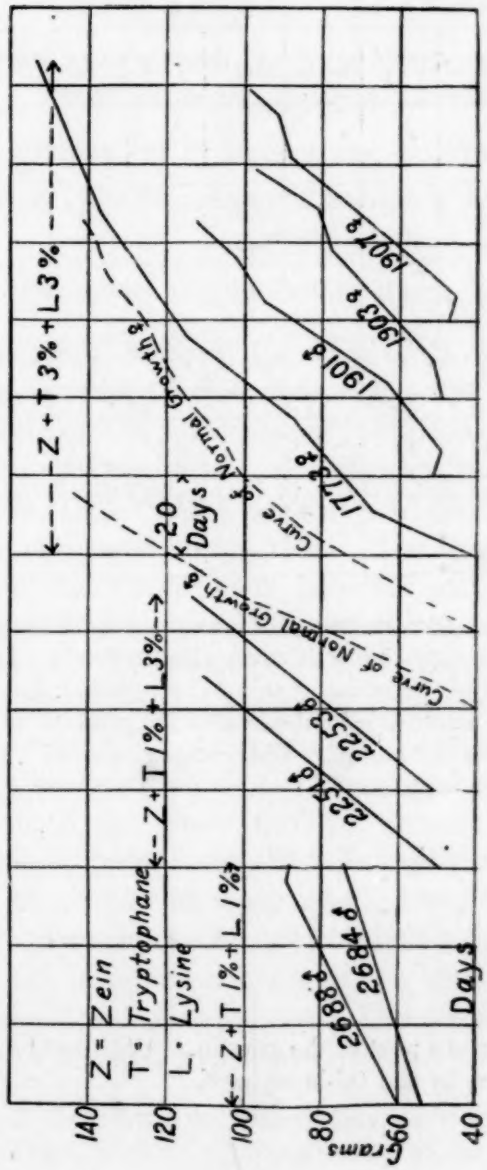

CHART III. Showing the proportions of tryptophane and lysine needed to promote good growth with zein foods. These records indicate clearly that to obtain maximal growth effects both amino-acids must be present in suitable quantities. The general composition of the foods used has been indicated in Chart I.

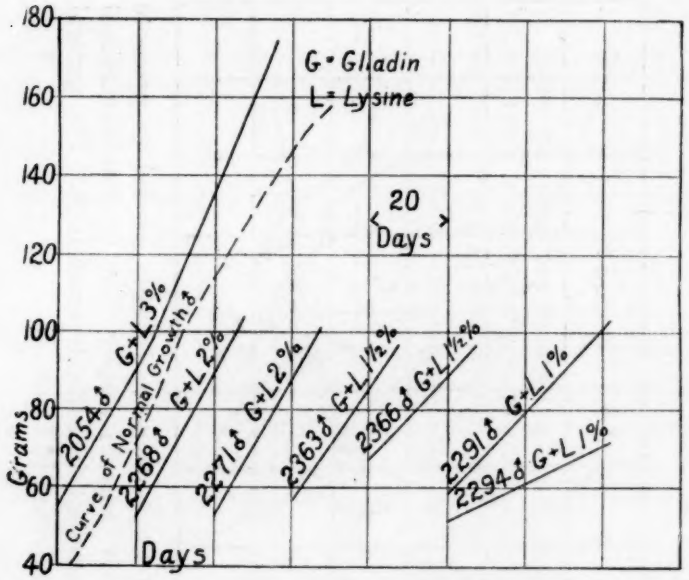

CHART IV. Showing the necessity of adequate additions of lysine in order to produce satisfactory growth upon gliadin foods. In considering the total lysine content of the ration it should be recalled that the gliadin—of itself inadequate to produce a food suitable for growth—is estimated to yield nearly 1 per cent of lysine. The composition of the ration was essentially as follows:

	per cent
Gliadin and lysine	18
"Protein-free" milk	28
Starch	22
Butter fat	18
Lard	14

The added lysine replaced a part of the gliadin. Additional data regarding these animals are given in the table on p. 6.

STUDIES IN THE SYNTHESIS OF HIPPURIC ACID IN THE ANIMAL ORGANISM.

III. THE EXCRETION OF URIC ACID IN MAN AFTER INGESTION OF SODIUM BENZOATE.

By HOWARD B. LEWIS and WALTER G. KARR.

(From the Laboratory of Physiological Chemistry of the University of Illinois, Urbana.)

(Received for publication, March 15, 1916.)

Much confusion exists in the vast mass of experimental data accumulated in recent years concerning the influence of benzoic acid or its salts on the nitrogenous metabolism of man. With regard to uric acid excretion, the greater number of observers[1] assert that the ingestion of benzoic acid has no effect, or results in a slightly increased elimination. The therapeutic use of benzoates in gout and rheumatism, however, still continues, although they have been replaced to a large extent by atophan and salicylic acid derivatives. That salicylic acid, closely related to benzoic, when administered causes a marked increase in the uric acid excretion, has repeatedly been shown.[2] Recently Denis[3] has reported three cases in which the relations between the uric acid concentration of the blood and the urinary elimination of uric acid were studied before and after the ingestion of 8 gm. of sodium benzoate. As a result of these studies Denis concludes: "Benzoic acid like salicylic acid causes, when taken in large doses, a marked decrease in the uric acid content of the blood and an increase in the uric acid content of the urine. The effect, however, is not so constant or so marked as with the former drug." From a care-

[1] Compare Keller, W., *Ann. Chem.*, 1842, xliii, 108. Lewandowsky, M., *Z. klin. Med.*, 1900, xl, 202. Chittenden, R. H., Long, J. H., and Herter, C. A., *U. S. Dept. Agric., Report No. 88*, 1909, pp. 58, 586.

[2] Rockwood, E. W., *Am. J. Physiol.*, 1909–10, xxv, 34. Fine, M. S., and Chace, A. F., *J. Biol. Chem.*, 1915, xxi, 371. Denis, W., *J. Pharm. and Exp. Therap.*, 1915, vii, 255.

[3] Denis, W., *J. Pharm. and Exp. Therap.*, 1915, vii, 601.

ful study of the figures presented, the conclusions in so far as they concern the urine would seem to be open to question, as in only one of the three cases is the increase beyond normal variations.

In connection with former work by one of us[4] on the rate of elimination of hippuric acid after benzoate ingestion, the observation was made that the uric acid content of the urine was very low in the periods immediately following the ingestion of the benzoate, those periods in which the elimination of the greater part of the hippuric acid takes place. It seemed probable that any variations in the uric acid excretion due to the benzoate ingestion would be evident if the elimination was studied over short periods of time, coincident with the periods of maximal hippuric acid excretion, and that relations which would be obscured in the normal variations of 24 hour periods might be observed. With this in view the following series of experiments were undertaken.

The subjects of the experiments were healthy young men, students or instructors in the laboratory. For at least 2 days prior to the experimental periods as well as during the experiment the subjects were maintained on a purine-free diet. The experimental periods consisted of 3 or 4 days. The urine was collected at regular 2 hour intervals throughout the day with a 12 hour period at night. No attempt was made to maintain the diet quantitatively uniform throughout the experiments. Each subject, however, partook of a uniform breakfast daily. The benzoate was dissolved in water and administered with the breakfast. Subject A ate no food prior to the midday meal and received the benzoate at 8 a.m. Uric acid was determined by Benedict and Hitchcock's[5] modification of the colorimetric method of Folin and Denis, a method which in our experience gives values checking closely with those obtained by the older method of Folin and Shaffer provided only 0.5 mg. of uric acid is used in the standard solution and diluted to 50 cc. Creatinine was determined by the method of Folin and total benzoic acid by the method of Folin and Flanders.

In order to see whether the presence of large quantities of hippuric acid in small volumes of urine, a condition met with in the experimental days of benzoate ingestion, in any way affected

[4] Lewis, H. B., *J. Biol. Chem.*, 1914, xviii, 225.

[5] Benedict, S. R., and Hitchcock, E. H., *J. Biol. Chem.*, 1915, xx, 619.

the quantitative determination of uric acid by the method employed, the following experiments were carried out.

1. To 25 cc. of one of the experimental urines of a normal day 0.6 gm. sodium hippurate was added and the uric acid determined in 5 cc. in comparison with the original urine.

	Original urine.	Urine after addition of sodium hippurate.
Colorimeter reading.....	20.8 mm.	21.8 mm. 22.0 " 21.7 " 21.8 " average of three determinations.
Uric acid calculated for total volume of period (400 cc.).....	48 mg.	45.8 mg.

2. The same procedure was followed with another urine, 0.6 gm. sodium hippurate being added as before.

	Original urine.	Urine after addition of sodium hippurate.
Colorimeter reading, average of three determinations.....	25.5 mm.	27.5 mm., average of four determinations.
Uric acid calculated for total volume of period (600 cc.).....	59 mg.	55 mg.

Repetition of the above experiments with different urines gave results similarly low, from 3 to 4 mg. lower than the results for the urine without the addition of sodium hippurate. It was observed that in the urine with the addition of the hippurate, the precipitate was less flocculent and less bulky than was the case with the original urine. The precipitate resembled that obtained with pure uric acid solutions, rather than the mixed precipitate of phosphates and uric acid obtained normally with urines by this method. We believe that the smaller bulk of the precipitate resulted in a less compact mass in the bottom of the centrifuge tube, which caused a slight loss when the supernatant liquid was poured off. We were unable to obtain the typical bulky flocculent precipitate even when large amounts of the ammoniacal silver magnesium solution were added. The quantities of hippurate added in the above experi-

ments were much greater than the amounts present in any of the experimental urines, so that the figures obtained above represent the maximum possible error under the conditions, with which we dealt in the present experiments. Any changes in the uric acid content of the urine following benzoate ingestion must then be well above any possible error of the method in the presence of hippuric acid, in order to be significant.

A study of Table II shows that *coincident with the maximal elimination of the ingested benzoate* (*during the first 4 hours after administration*, compare Table VI), *the uric acid excretion is markedly diminished, a decrease of 50 to 70 per cent as compared with the excretion during the corresponding periods of the fore and after days.* These decreases while marked in the shorter periods are obscured in the figures for 24 hours, in which, although decreased elimination is evident, the variations are hardly more than normal fluctuations. This is especially marked in the experiments with Subjects A and L. When, however, the results for the first 4 hours of the day are summarized (Table I), the variations are seen to be well above any normal fluctuations or experimental error.

TABLE I.

Elimination of Uric Acid and Creatinine during the First 4 Hours of the Day.

Subject.	Day.	Uric acid.	Creatinine.	Sodium benzoate administered.
		mg.	*mg.*	*gm.*
H. B. L.	Fore	140	311	0
	Fore	150	305	0
	Benzoate	**71**	**293**	**8**
	After	142	341	0
W. G. K.	Fore	128	402	0
	Fore	122	313	0
	Benzoate	**47**	**354**	**8**
	After	152	365	0
L. A.	Fore	89	290	0
	Benzoate	**34**	**261**	**7**
	After	118	299	0

Two possible explanations of this decrease suggest themselves. The changes might be due to a delayed elimination of the uric acid

by the kidneys, a retardation which should be followed by a compensatory increase in the later periods of the day. An inspection of the tables shows that no increased elimination in any way comparable to the decreases in the elimination of the earlier periods or greater than the normal variations of the fore and after days occurred. The possibility that these changes are the result of a general lowering of the plane of endogenous metabolism due to the toxic action of benzoic acid must also be taken into consideration. In order to test this hypothesis, creatinine was also determined throughout the experiments. No changes in the elimination of creatinine were observed (Tables I and IV). If the decreased excretion of uric acid observed is the result of a decreased formation of uric acid due to lowering of the level of nuclear metabolism under the influence of sodium benzoate, this must be considered as a specific influence on nuclear metabolism rather than on general endogenous metabolism, since the creatinine elimination remains normal. Whether the gastro-intestinal irritation commonly ascribed to the influence of large doses of benzoic acid would cause an increase in the endogenous uric acid metabolism is not clear from our present knowledge.[6] Certainly there is no evidence of the opposite effect.

The ingestion of benzoic acid results in the production of glycocoll from sources as yet unknown, for purposes of detoxication. Is it not possible that uric acid may be broken down to yield glycocoll or some other substance for detoxication, or may even itself be conjugated with benzoic acid? Uric acid is present normally in small amounts in human blood and tissues (1 to 2 mg. per 100 cc. of blood according to Folin and Denis[7]), while proteins and amino-acids, in the last analysis the sources of glycocoll, occur in much larger amounts. If then, as has been suggested, uric acid contributes in some unknown way to the detoxication of benzoic acid, one would expect the reactions to proceed according to the masses of substances available, the greater rôle in the detoxication being played by the substance most abundant; *i.e.*, glycocoll and its precursors to yield hippuric acid, and the less abundant uric acid playing a less important rôle in detoxication.

[6] Mendel, L. B., and Stehle, R. L., *J. Biol. Chem.*, 1915, xxii, 215.
[7] Folin, O., and Denis, W., *J. Biol. Chem.*, 1913, xiv, 29.

In connection with this problem it seemed also of interest to determine whether this effect of benzoic acid on uric acid excretion was also produced by hippuric acid administered *per os*. Is the effect of the benzoic acid due to a toxic action on the cells, resulting in a decreased formation of uric acid, to the utilization of the uric acid formed for detoxication, or to the presence of hippuric acid circulating in the system? The experiments were conducted in the same manner as those previously described in this paper. An amount of sodium hippurate equivalent to the benzoate fed in the first series was administered.

As seen in Tables III and V, no significant changes in the elimination of creatinine or uric acid resulted from the administration of large doses of sodium hippurate. The decreased uric acid excretion observed after benzoate ingestion would seem to be due to the effect of the benzoic acid itself and not to its conjugated, non-toxic derivative, hippuric acid.

Notable also in the series of experiments is the slower elimination of the hippuric acid after the administration of sodium hippurate, than after benzoate administration (Table VI).

TABLE II.

The Influence of Benzoate Ingestion on Uric Acid Excretion.

Subject.	Day.	Sodium benzoate fed.	Uric acid in mg. excreted for period ending							Total uric acid in 24 hrs.
			9 a. m.	11 a. m.	1 p. m.	3 p. m.	5 p. m.	7 p. m.	7 a. m.	
		gm.								*mg.*
H. B. L. Wt. 86 kg.	1	0	62	78	53	64	50	50	300	657
	2	0	75	75	64	65	56	34	210	579
	3	**8**	**30**	**41**	52	66	46	45	255	**535**
	4	0	78	64	62	74	43	Lost.	212	.....
W. G. K. Wt. 74 kg.	1	0	55	73	58	45	52	53	239	575
	2	0	55	67	63	42	53	47	228	555
	3	**8**	**28**	**19**	43	32	36	39	259	**456**
	4	0	78	74	63	61	53	48	166	543
L. A.* Wt. 59.1 kg.	1	0	55	34	32	36	39	47	212	455
	2	**7**	**16**	**18**	49	37	40	54	206	**420**
	3	0	57	61	46	47	50	52	203	516

* Collection of urine beginning at 8 a.m. in 2 hr. periods.

TABLE III.

The Influence of Hippurate Ingestion on Uric Acid Excretion.

Subject.	Day.	Sodium hippurate fed.	Uric acid in mg. excreted for period ending							Total uric acid in 24 hrs.
			9 a. m.	11 a. m.	1 p. m.	3 p. m.	5 p. m.	7 p. m.	7 a. m.	
		gm.								*mg.*
H. B. L.	1	0	89	70	63	68	45	40	285	660
Wt.	2	**11.2**	**65**	**58**	**46**	**69**	65	Lost.	305	.....
86 kg.	3	0	72	68	48	43	46	32	216	525
W.G.K.	1	0	65	67	49	48	44	31	239	543
Wt.	2	**11.2**	**60**	**81**	**51**	**75**	54	41	246	**608**
74 kg.	3	0	78	66	42	47	31	37	226	527

TABLE IV.

The Influence of Benzoate Ingestion on Creatinine Excretion.

Subject.	Day.	Sodium benzoate fed.	Creatinine in mg. excreted for period ending							Total creatinine in 24 hrs.
			9 a. m.	11 a. m.	1 p. m.	3 p. m.	5 p. m.	7 p. m.	7 a. m.	
		gm.								*mg.*
H. B. L.	1	0	149	162	143	162	126	144	810	1696
Wt.	2	0	168	137	155	143	158	116	810	1687
86 kg.	3	8	163	130	144	162	137	117	730	1583
	4	0	205	136	148	158	135	Lost.	675	.....
W.G.K.	1	0	189	213	182	171	197	171	1088	2211
Wt.	2	0	176	137	164	176	247	187	894	1981
74 kg.	3	8	193	161	174	202	127	154	988	1999
	4	0	186	179	194	177	177	178	868	1959
L. A.*	1	0	162	128	125	147	180	142	810	1694
Wt.	2	7	140	121	162	127	174	150	765	1639
59.1 kg.	3	0	152	147	142	130	165	147	837	1720

* Collection of urine beginning at 8 a.m. in 2 hr. periods.

TABLE V.

The Influence of Hippurate Ingestion on Creatinine Excretion.

Subject.	Day.	Sodium hippurate fed.	Creatinine in mg. excreted for period ending							Total creatinine in 24 hrs.
			9 a. m.	11 a. m.	1 p. m.	3 p. m.	5 p. m.	7 p. m.	7 a. m.	
		gm.								*mg.*
H. B. L.	1	0	248	169	169	179	145	168	965	2043
Wt.	2	11.2	162	162	162	173	169	Lost.	980	.....
86 kg.	3	0	156	152	159	147	170	202	1039	2025
W. G. K.	1	0	197	195	202	196	202	152	1100	2244
Wt.	2	11.2	193	187	170	225	206	188	1048	2217
74 kg.	3	0	219	208	190	221	170	163	1318	2489

TABLE VI.

Total Benzoic Acid Excretion following Ingestion of Sodium Benzoate or Sodium Hippurate.

Subject.	Day.	Sodium benzoate or sodium hippurate fed calculated as benzoic acid.	Total benzoic acid in gm. excreted during period ending				Total benzoic acid in 8 hrs.
			9 a. m.	11 a. m.	1 p. m.	3 p. m.	
		gm.					*gm.*
H. B. L.....	3	Benzoate. 6.76	2.94	2.86	0.45	0.16	6.41
H. B. L.....	2	Hippurate. 6.76	2.56	1.91	0.54	0.51	5.52
W. G. K.....	3	Benzoate. 6.76	1.96	2.57	1.48	0.21	6.22
W. G. K.....	2	Hippurate. 6.76	1.09	2.80	1.34	0.72	5.95
L. A.*.....	2	Benzoate. 5.92	1.95	2.39	1.39	0.14	5.87

* Collection of urine beginning at 8 a.m. in 2 hr. periods.

STUDIES IN THE BLOOD RELATIONSHIP OF ANIMALS AS DISPLAYED IN THE COMPOSITIONS OF THE SERUM PROTEINS.

V. THE PERCENTAGE OF NON-PROTEINS IN THE SERA OF CERTAIN ANIMALS AND BIRDS.

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(Received for publication, March 13, 1916.)

This investigation was undertaken with a view to correcting and extending the figures given in former articles of this series by adding the determination of non-proteins for each animal.

In former articles of this series[1] it was assumed that the amount of non-proteins in the blood sera of all animals was about the same; but it has since been ascertained that this assumption is not accurate;[2] and the present work was undertaken to ascertain the amount of non-protein in the blood of each kind of animal formerly studied and to correct the figures which are affected by the revised estimation of the non-proteins. In the present investigation Robertson's refractometric method[2] was used in so far as the determination of non-proteins is concerned, and in cases where the concentration of the serum being examined was much greater or less than that examined by former investigators, the ascertained amount of non-protein was increased or reduced in like proportion before correcting the previously published figures.

The following table shows the refractive indices obtained, the percentages of albumin, globulin, and total proteins determined by previous investigators, and the figures as corrected by the present investigation. Where the percentage of non-protein in

[1] Robertson, T. B., *J. Biol. Chem.*, 1912, xi, 179; 1912–13, xiii, 325. Woolsey, J. H., *ibid.*, 1913, xiv, 433. Thompson, W. B., *ibid.*, 1915, xx, 1. Briggs, R. S., *ibid.*, 1915, xx, 7.

[2] Robertson, *J. Biol. Chem.*, 1915, xxii, 233.

the seventh column of figures differs from that in the first column, that in the first column is the figure obtained from the serum investigated and that in the seventh column is the proportional figure calculated to correspond to the concentration of the serum used by the former investigator. One to three determinations were made in each case according to the amount of blood obtainable. The animals employed were normal animals, not previously fasted.

Animal.	Given in former articles.						Corrected by present investigations.					
	Non-protein.	Globulin.	Albumin.	Total protein.	As percentage of total protein.		Non-protein.	Globulin.*	Albumin.	Total protein.	As percentage of total protein.	
					Globulin.	Albumin.					Globulin.	Albumin.
	per cent	*per cent*	*per cent*	*per cent*			*per cent*	*per cent*	*per cent*	*per cent*		
Horse	1.51	3.5	4.6	8.1	43	57	1.65	3.5	4.0	7.5	47	53
2 albino rats	1.61	1.7	4.8	6.5	26	74	1.61	1.7	4.23	5.93	29	71
Ox	1.34	2.21	5.32	7.50	29	70	1.34	2.21	5.00	7.21	31	69
Hog	1.62	2.76	4.86	7.60	36	64	1.49	2.76	4.34	7.10	39	61
Sheep	1.30	1.14	5.44	6.59	17	82	1.30	1.14	5.15	6.29	18	82
Goat	1.43	1.56	5.31	6.87	22	75	1.43	1.56	4.90	6.46	24	76
Cat	1.73	2.56	5.86	8.42	30	69	1.87	2.56	5.06	7.62	34	66
Dog	2.01	1.31	5.76	7.07	18	81	2.01	1.31	4.82	6.13	21	79
Guinea pig	1.53	0.93	5.01	5.94	16	84	1.28	0.93	4.74	5.67	16	84
Hen	1.81	1.14	3.9	5.0	23	77	1.42	1.14	3.50	4.64	25	75
Duck	1.90	1.91	4.8	6.7	26	74	2.77	1.91	3.18	5.09	38	62

* Globulin unchanged.

THE TOXIC ACTION OF SOLUBLE ALUMINIUM SALTS UPON THE GROWTH OF THE RICE PLANT.

By K. MIYAKE.

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(Received for publication, March 7, 1916.)

The matter of soil acidity is one of the live topics of the day among soil investigators. There has been a vast amount of investigation of the subject[1] and it has come to be quite generally recognized in recent years that by the addition of various kinds of salt solutions to soils, soluble acids or acid-acting salts are set free: that is, cultivated soils tend to become acid year by year as a consequence of the application of manures and fertilizers containing soluble salts, because when nitrates, chlorides, and sulfates are added to soils, the tendency is for the base to combine with the organic matter and the silicates in the soil, and for the acid radicle to combine with aluminium and to a less extent with iron. The soluble aluminium and iron salts which are formed are more or less

[1] Among the numerous contributors to the literature on the subject may be mentioned:

Van Bemmeln, J. M., *Ber. chem. Ges.*, 1878, xi, 2228; *Landw. Vers.-Stat.*, 1888, xxxv, 69.

Hopkins, C. G., Pettit, J. H., and Knox, W. H., *U. S. Dept. of Agriculture, Bureau of Chemistry, Bull. 73*, 1903, 114.

Veitch, F. P., *J. Am. Chem. Soc.*, 1904, xxvi, 637.

Kohler, E., *Z. prakt. Geol.*, 1903, xi, 49.

Sullivan, E. C., *U. S. Geol. Survey, Bull. 312*, 1907.

Wiegner, G., *J. Landw.*, 1912, lx, 111, 197.

Cameron, F. K., The Soil Solution, Easton, Pa., 1911.

Parker, E. G., *J. Agric. Research*, 1913–14, i, 179.

Gans, R., *Internat. Mitt. Bodenk.*, 1913, iii, 529.

Loew, O., *Porto Rico Experiment Station, Bull. 13*, 1913.

Harris, J. E., *J. physik. Chem.*, 1914, xviii, 355.

Daikuhara, G., *Bull. Imperial Centr. Agric. Experiment Station, Japan*, 1914, ii, 1.

Conner, S. D., *J. Ind. and Eng. Chem.*, 1916, viii, 35.

hydrolyzed in solution, and the consequence is the setting free of soluble acid; that is, the presence of excess of acid in the soil. Of course the acid radicles combine with the stronger bases, such as calcium and magnesium, but the acid condition of any soil is due to the fact that it does not have a sufficient supply of the strong bases; hence in many soils aluminium and iron necessarily supply the basic radicle. It thus happens that in many cases the apparent acidity of the extract from the soil as determined by titration with standard alkali in the presence of phenolphthalein, is found to be proportional to the amount of aluminium salts present in the solution, and evidently represents the amount of alkali required to precipitate the aluminium rather than actual free acidity. Accordingly, the addition of strong bases as calcium to the soil or treatment such as ignition which makes soil constituents less reactive, will stop or decrease the production of acid by the application of soluble salts to the soils.

From the view of soil acidity above stated, it appeared to be of interest to investigate the toxicity of soluble aluminium and iron salts and its relation to their acidity. As the soluble iron salts are generally present in negligible traces in the soil solution, we will confine our investigation to aluminium salts.

There are a few publications on the physiological act on of aluminium salts on the growth of plants. Micheels and De Heen investigated the effect of aluminium salts on the germination of wheat, with the result that, while they found alumina or kaolin to be beneficial, the addition of soluble aluminium salts proved injurious. House and Gies[3] found that the toxicity of aluminium salts is dependent entirely upon the concentration. Yamano[4] ascertained by pot experiment on barley and flax that moderate amounts o aluminium salts have a stimulating action on plant development. In water cultures, however, 0.2 per cent of alum acted injuriously after 3 weeks, while 0.8 per cent killed the plant in a few days. Hébert[5] studied the toxicity of chromium and aluminium sulfates in comparison with the sulfates of some of the rare metals. Progressively increasing amounts of these compounds were tested on germinated seeds of pea, wheat, and rape. 'Aluminium, chromium, and some of the rare metal sulfates were found to be strongly poisonous. Duggar[6] reports aluminium

[2] Micheels, H., and De Heen, P., *Bull. Acad. roy. Belg.*, 1905, 520.
[3] House, H. D., and Gies, W. J., *Am. J. Physiol.* 1905-06, xv, p. xix.
[4] Yamano, Y., *Bull. College Agric., Tokyo Imperial Univ.*, 1905, vi, 429.
[5] Hébert, A., *Bull. Soc. chim.*, 1907, series 4, i, 1026.
[6] Duggar, B. M., Plant Physiology, New York, 1911.

chloride and sulfate extremely toxic to pea grown in water cultures. Abbott, Conner, and Smalley[7] found that very dilute solutions of aluminium nitrate are toxic to corn seedlings in water culture in the presence of mineral nutrients. The toxicity of aluminium nitrate was found to be approximately equal to that of nitric acid of the same normality. E. Kratzmann[8] proved that the growth of higher plants is retarded by the presence of 0.05 per cent of aluminium salts, but very small amounts (0.0001 per cent) have a slight stimulating action. Ruprecht[9] found also that aluminium sulfate, when present in culture solutions in concentrations of aluminium greater than forty parts per million, has a very toxic action on clover seedlings.

From the above review it will be seen that, although there is evidence that aluminium salts are toxic, there has been no research work done on the question of the relationship of the toxicity of aluminium salts to their acidity, except the article by Abbott, Conner, and Smalley which touches on this matter.

Water cultures were employed in the investigation. Though of limited value in soil fertility investigation from the standpoint of agriculture, this method of investigation furnishes an excellent means of studying special phases in which physiological effects are important. For the solution I used aluminium chloride in concentrations of $\frac{N}{1,000}$ to $\frac{N}{20,000}$ as will be seen in Table I, and hydrochloric acid solutions of same concentrations were used for comparison. In the experiment I started with the young rice seedlings, 10 mm. high, which were grown in distilled water from seeds of almost uniform size and specific gravity. Fourteen beakers about 5.5 cm. in diameter and 7 cm. deep, each containing 30 cc. of solution, were used for the experiment, the seedlings being placed in the solution, while one beaker containing distilled water served as a control. Five seedlings were grown in each culture at ordinary temperature and the evaporated water was supplemented with distilled water from time to time to keep the solutions always at their initial dilutions. After 13 days, the difference in development was striking, when the following measurements were made.

[7] Abbott, J. B., Conner, S. D., and Smalley, H. R., *Ind. Experiment Station, Bull. 170*, 1913, 329.

[8] Kratzmann, E., *Chem. Ztg.*, 1914, xxxviii, 1040.

[9] Ruprecht, R. W., *Mass. Experiment Station, Bull. 161*, 1915, 125.

TABLE I.

		Concentration.							
		$\frac{N}{1,000}$	$\frac{N}{2,500}$	$\frac{N}{5,000}$	$\frac{N}{7,500}$	$\frac{N}{10,000}$	$\frac{N}{15,000}$	$\frac{N}{20,000}$	Control.
$AlCl_3$	Length of leaf, mm..	50	51	51	55	57	57	57	57
	Length of root, mm..	12	12	16	26	36	36	52	52
	No. of roots.....	5*	8*	8*	8	7	7	7	7
HCl	Length of leaf, mm...	41	48	48	55	57	57	62	57
	Length of root, mm...	26	33	33	56	55	52	52	52
	No. of roots.....	1	2*	7*	7	7	7	7	7

* Only the primary root was well developed

Though a little difference of growth between the aluminium chloride and hydrochloric acid solutions was observed, the table shows that each solution in the same normality causes almost similar retardation as stated by Abbott, Conner, and Smalley, and concentrations greater than $\frac{N}{7,500}$ are injurious to the growth of rice seedlings. From this result we cannot immediately conclude that the toxic action of aluminium chloride is due to the hydrogen ions formed by the hydrolysis of the salt in solution unless we have a reliable measurement of the hydrogen ion concentration in aluminium chloride solutions, because, though aluminium chloride is hydrolyzed in solution, we cannot consider that the hydrolysis is complete and that the concentration of hydrogen ion is the same as that in a solution of hydrochloric acid of the same normality.

An examination of the literature failed to reveal any data on the degree of hydrolysis or concentration of hydrogen ions of aluminium chloride solutions. I have, therefore, measured the concentration of hydrogen ions in some of the above mentioned solutions of aluminium chloride by the method of the gas chain. Details of the apparatus and method of manipulation are now too well known to require description. For the determination I had the assistance and apparatus of Mr. C. L. A. Schmidt, to whom I express my thanks. The concentration of hydrogen ions was measured in the solution of $\frac{N}{7,500}$ aluminium chloride, which seems to be a critical concentration of toxicity, and in two other dilutions, that is, $\frac{N}{1,000}$ and $\frac{N}{5,000}$, which are toxic to the growth. The

result obtained is as follows. The concentration of hydrogen ion of hydrochloric acid in same normality was also calculated under the supposition that the hydrochloric acid is completely dissociated into hydrogen and chlorine ions in such dilutions.

TABLE II.

	Concentration.		
	$\frac{N}{1.000}$	$\frac{N}{5,000}$	$\frac{N}{7,500}$
HCl	1.000×10^{-3}	2.000×10^{-4}	1.330×10^{-4}
$AlCl_3$	0.317×10^{-3}	0.716×10^{-4}	0.505×10^{-4}
$HCl : AlCl_3$	1 : 0.317	1 : 0.358	1 : 0.379

Though the results obtained by the determination are not very accurate, because the measurement was very difficult owing to the high internal resistance of such solutions, still the great differences between the hydrogen ion concentration in the aluminium chloride and hydrochloric acid solutions could not be attributed to the experimental error. From this result it may therefore be safely concluded that the actual acidity of aluminium chloride solutions is less than that of hydrochloric acid. The toxicity of aluminium chloride, as we have seen, however, appears to be almost exactly the same as that of hydrochloric acid of the same normality. It is, therefore, clear that the acidity of aluminium chloride solutions is not the cause of the toxic action of the salt. The toxic effect of aluminium chloride solution must be due to other factors.

SUMMARY.

Taking the above results into consideration we may safely formulate the following conclusions:

1. Aluminium chloride is toxic to the growth of rice seedlings even in dilute solution.

2. The toxic effect of aluminium chloride upon the growth of rice seedlings appears in concentrations greater than $\frac{N}{7,500}$.

3. The toxicity of aluminium chloride seems to be approximately equal to that of hydrochloric acid of the same normality.

4. The toxic action of aluminium chloride is not due to the hydrogen ion formed by hydrolysis of the salt in solution.

5. The concentration of hydrogen ions formed by the hydrolysis of aluminium chloride is less than that formed by dissociation of hydrochloric acid of the same normality.

6. Since the chlorine ion is not toxic to the growth of rice seedlings in such dilute solution,[10] colloidal aluminium hydroxide or unhydrolyzed aluminium chloride molecules or aluminium ions may be the toxic factors.

7. The toxicity of soluble aluminium salts is dependent upon the amount of aluminium itself. The determination of soil acidity by titration in which the soil extract is titrated with standard alkali is a logical method of determining the amount of bases which should be added to the soil for the amelioration of its infertility; because, although the titration does not indicate the true acidity of the soil, yet it does afford a measure of the bases which must be added to neutralize the free acid and decompose the aluminium salts, either or both of which may be responsible for the infertility.

In conclusion, the author wishes to express his thanks to Dr. T. Brailsford Robertson for his kind advice during this experiment.

[10] Miyake, *K.*, *Tr. Sapporo Natural History Soc.*, Japan, 1914, v, 91.

SOLUBILITY DATA FOR VARIOUS SALTS OF LAURIC, MYRISTIC, PALMITIC, AND STEARIC ACIDS.*

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(Received for publication, March 15, 1916.)

The isolation of individual members of the higher saturated fatty acids, when occurring in mixtures of such acids, has always been fraught with considerable, if not insurmountable difficulties.

The authors, in their characterization of the constituents of alfalfa seed oil,[1] were confronted with the same difficulties. After applying all the known methods that were available for these separations, and finding that unsatisfactory results were obtained in every case when artificially prepared mixtures of the fatty acids were employed, it was decided to investigate the solubilities of various salts of lauric, myristic, palmitic, and stearic acids in a number of organic solvents as well as in water.

It was hoped that the solubility data thus obtained would furnish the means for a scheme of separation of the different acids, but just how far these anticipations were realized will be discussed in another paper.

The property of solubility has furnished the basis for more systems of separation of chemical substances than any other, although differences of melting point, boiling point, and volatilization in steam are properties upon which schemes of separation have been founded.

The following tables include the solubility of the lithium, magnesium, beryllium, barium, lead, and silver salts of lauric, myristic, palmitic, and stearic acids in two or more of the following solvents: water, ethyl and methyl alcohol, ether, benzene, ethyl acetate, methyl acetate, amyl alcohol, amyl acetate, chloro-

* This investigation was carried out at the Nevada Agricultural Experiment Station with funds obtained under the Adams Act.

[1] Jacobson, C. A., and Holmes, A., *J. Am. Chem. Soc.*, 1916, xxxviii, 480.

form, and acetone, at room temperature, 25°, 35°, and 50°, whenever the boiling point of the solvent permitted.

The solvents were selected according to their most probable applicability along this line. They were of the highest purity obtainable and always redistilled whenever any question as to their purity arose. In general the salts of the fatty acids were made from the acetates of the metals, but a detailed description of the preparation of each salt will be given in connection with its table of solubilities.

An excess of the salt to be investigated was put into a 100 cc. round bottom flask having a neck about 5 inches long. Four such flasks were used at the same time for the four different salts of a given metal and the flasks filled nearly full with the solvent to be employed. The flasks were then stoppered and clamped in a shaking device, which was so arranged that the flasks, excepting the upper part of the necks, were immersed in a bath of water whose temperature was kept constant to within 0.5°. An electric motor was used for shaking the flasks in the water bath and the agitation continued for 2 hours after the liquid in the flasks had assumed the temperature of the water in the bath. The shaking was then interrupted and the suspended matter allowed to settle, after which about 10 cc. of the solution were rapidly drawn off with a carefully calibrated pipette, the solution was weighed in a covered weighing tube, and finally the solvent evaporated off. From the weight of the residue in the weighing tubes the solubility was calculated in terms of gm. of salt, soluble in 100 gm. of solvent.

We did not attempt to obtain absolute solubilities in any case, although most of the data here submitted will approach the absolute values very closely. It has been learned that in some instances a 6 hour shaking in contact with the solvent is not sufficient to secure maximum solubility, but for the solvents used the difference between the solubility after 2 and 6 hours' shaking is so slight that for all practical purposes it may be neglected. Amyl alcohol may be considered an exception to this rule for it was found that a 2 hour shaking in most cases did not suffice to produce a saturated solution. The results recorded under this solvent are those obtained after 2 hours' shaking, making them comparable with those of the other salts.

In the first table will be found the results of analyses of the four fatty acids and their salts which were employed in obtaining the following solubility data.

TABLE I.

	Lauric acid.	Myristic acid.	Palmitic acid.	Stearic acid.
M. p. found	43.0°	49.7°	61.4°	69 0°
M. p. given	43.6°	53.8°	62.0°	69.2°
Neutralization value found	280.0	228.5	220.4	199.3
Neutralization value calculated	280.5	246.1	219.1	197.5
M. p. Li salt	229.2–229.8°	223.6–224.2°	224–225°	220.5–221.5°
Per cent of Li in Li salt found	3.44	2.91	2.58	2.38
Per cent of Li calculated	3.48	2.99	2.66	2.41
M. p. Mg salt	150.4°	131.6°	121–122°	132°
Per cent of Mg in Mg salt found	5.73	5.09	4.51	4.20
Per cent of Mg calculated	5.75	5.08	4.54	4.11
M. p. Pb salt	104.6–104.8°	108.6–108.8°	112.2–112.4°	115.6–115.8°
Per cent of Pb in Pb salt found	33.66	31.01	28.24	26.50
Per cent of Pb calculated	34.28	31.31	28.86	26.77
Per cent of Ba in Ba salt found	25.82	24.20	21.62	19.60
Per cent of Ba calculated	25.64	23.22	21.20	19.52
M. p. Ag salt	212–213°	211°	209°	205°
Per cent of Ag in Ag salt found	34.78	32.45	29.74	28.05
Per cent of Ag calculated	35.12	32.20	29.72	27.58

In Table II will be found the analyses of the beryllium salts of the four fatty acids which were prepared in the following manner: To 10 gm. of the acid dissolved in 95 per cent alcohol (the solution neutralized with alcoholic ammonium hydroxide, using litmus as the indicator), a calculated amount of beryllium nitrate dissolved in alcohol was added. The resulting precipitate was

washed several times with boiling alcohol, both by decantation and in a filter, then dried and subjected to analysis with the following results.

TABLE II.

	Be laurate.	Be myristate.	Be palmitate.	Be stearate.
Per cent of Be found	3.71	3.48	3.08	2.81
Calculated for Be $(Ac)_2$	2.23	1.96	1.75	1.58
Ratio: $\frac{\text{Found}}{\text{Calculated}}$	1.66	1.77	1.76	1.78
Hence, calculated for Be(OH)Ac	4.04	3.59	3.23	2.87
Per cent of C found	63.85	66.19	67.95	70.17
Calculated for Be(OH)Ac	63.91	66.33	68.23	69.81
Per cent of H found	10.93	10.90	11.53	11.91
Calculated for Be(OH)Ac	10.74	11.13	11.48	11.73

The above results show that the basic salts of beryllium with the formula Be(OH)Ac were obtained rather than the normal $Be(Ac)_2$ salts. They were found to be only very slightly soluble in the general organic solvents, but for the sake of completeness their solubilities were determined in ethyl and methyl alcohol at 25°. Table III contains these data.

TABLE III.

Solubility of ~~Acid~~ Basic Salts of Beryllium at 25°.

	Laurate.	Myristate.	Palmitate.	Stearate.
Ethyl alcohol $\frac{\text{gm. salt}}{\text{100 gm. solvent}}$	0.004	0.004	0.004	..
Methyl alcohol $\frac{\text{gm. salt}}{\text{100 gm. solvent}}$	0.050	0.047	0.042	0.040

Table IV contains the solubility data for the lithium salts prepared by adding a calculated amount of lithium acetate to the alcoholic solutions of the respective fatty acids. The precipi-

tates formed were dissolved in boiling alcohol and the solutions allowed to stand over night in a cool place. The salts that had separated were washed and dried.

TABLE IV.

Solubility of Lithium Salts in Ethyl Alcohol (Absolute).

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
		Temperature 20°		
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0313	7.79	7.76	0.403
Myristate	0.0147	7.57	7.55	0.194
Palmitate	0.0075	7.81	7.80	0.096
Stearate	0.0056	7.74	7.73	0.072
		Temperature 25.4°		
Laurate	0.0342	7.69	7.66	0.447
Myristate	0.0174	7.77	7.76	0.224
Palmitate	0.0092	7.80	7.79	0.118
Stearate	0.0060	7.78	7.78	0.089
		Temperature 35°		
Laurate	0.0418	7.69	7.65	0.546
Myristate	0.0215	7.74	7.72	0.278
Palmitate	0.0110	7.77	7.76	0.142
Stearate	0.0082	7.73	7.72	0.106
		Temperature 50°		
Laurate	0.0594	7.66	7.60	0.782
Myristate	0.0335	7.69	7.66	0.435
Palmitate	0.0190	7.64	7.61	0.248
Stearate	0.0154	7.69	7.67	0.200
		Temperature 65°		
Laurate	0.0827	7.28	7.20	1.149
Myristate	0.0490	7.38	7.33	0.669
Palmitate	0.0306	7.85	7.82	0.391
Stearate	0.0256	7.72	7.69	0.333

The solubilities here recorded are practically the absolute solubilities of the salts used, if the results of Partheil and Ferié[2] can be taken to represent absolute solubilities.

TABLE V.

Solubility of Lithium Salts in Methyl Alcohol.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 15.2°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.2442	7.97	7.73	3.159
Myristate	0.1055	7.95	7.84	1.346
Palmitate	0.0486	7.94	7.89	0.616
Stearate	0.0321	9.23	9.20	0.349
Temperature 25°				
Laurate	0.2883	7.93	7.64	3.773
Myristate	0.1299	7.86	7.73	1.680
Palmitate	0.0604	7.89	7.83	0.771
Stearate	0.0344	7.85	7.82	0.439
Temperature 34.6°				
Laurate	0.3463	7.88	7.53	4.597
Myristate	0.1684	7.85	7.68	2.193
Palmitate	0.0850	7.82	7.83	1.086
Stearate	0.0513	7.84	7.79	0.658
Temperature 50°				
Laurate	0.4487	7.82	7.37	6.088
Myristate	0.2329	7.49	7.25	3.281
Palmitate	0.1252	7.65	7.52	1.652
Stearate	0.0810	7.76	7.68	1.057

[2] Partheil, A., and Ferié, F., *Arch. Pharm.*, 1903, ccxli, 545.

TABLE VI.

Solubility of Lithium Salts in Water.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 16.3°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0152	9.86	9.84	0.154
Myristate	0.0027	9.85	9.85	0.027
Palmitate	0.0010	9.84	9.84	0.010
Stearate	0.0009	9.86	9.86	0.009
Temperature 25°				
Laurate	0.0184	9.86	9.84	0.187
Myristate	0.0039	10.83	10.83	0.036
Palmitate	0.0015	9.85	9.85	0.015
Stearate	0.0010	9.83	9.83	0.010
Temperature 35°				
Laurate	0.0203	9.83	9.81	0.207
Myristate	0.0042	10.01	10.02	0.042
Palmitate	0.0015	9.83	9.82	0.015
Stearate	0.0010	9.12	9.12	0.010
Temperature 50°				
Laurate	0.0274	9.81	9.78	0.280
Myristate	0.0061	9.79	9.79	0.062
Palmitate	..	..	..	..
Stearate	..	..	..	..

TABLE VII.

Solubility of Lithium Salts in Ether.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 15.8°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0008	7.20	7.20	0 011
Myristate	0.0009	7.00	7.00	0.013
Palmitate	0.0005	7.09	7.09	0.007
Stearate	0.0008	7.23	7.23	0.011
Temperature 25°				
Laurate	0.0005	7.98	7.98	0.006
Myristate	0.0003	7.73	7.73	0.004
Palmitate	0.0006	8.35	8.35	0.007
Stearate	0.0008	7.01	7.01	0.011

TABLE VIII.

Solubility of Lithium Salts in Amyl Alcohol.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 16°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0058	7.92	7.92	0.073
Myristate	0.0024	8.21	8.21	0.029
Palmitate	0.0016	8.33	8.32	0.019
Stearate	0.0008	7.45	7.45	0.011
Temperature 25.7°				
Laurate	0.0081	7.29	7.28	0.111
Myristate	0.0036	7.74	7.74	0.046
Palmitate	0.0024	7.48	7.47	0.032
Stearate	0.0022	7.88	7.88	0.028
Temperature 35°				
Laurate	0.0101	8.03	8.02	0.126
Myristate	0.0052	8.33	8.33	0.062
Palmitate	0.0028	8.37	8.37	0.033
Stearate	0.0024	7.86	7.86	0.031
Temperature 49.2°				
Laurate	0.0173	8.54	8.53	0.203
Myristate	0.0079	7.22	7.21	0.109
Palmitate	0.0052	7.49	7.48	0.069
Stearate	0.0046	7.61	7.61	0.060

TABLE IX.

Solubility of Lithium Salts in Chloroform.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 15.2°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0010	16.59	16.59	0.006
Myristate	0.0007	16.03	16.03	0.004
Palmitate	0.0006	15.60	15.60	0.004
Stearate	0.0007	16.37	16.37	0.004

TABLE X.

Solubility of Lithium Salts in Amyl Acetate.

	Salt.	Solution.	Solvent.	Salt in 100 gm solvent.
Temperature 14.5°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0056	8.23	8.23	0.068
Myristate	0.0031	8.40	8.40	0.037
Palmitate	0.0023	6.07	6.07	0.038
Stearate	0.0028	8.13	8.13	0.034
Temperature 25°				
Laurate	0.0052	8.07	8.07	0.064
Myristate	0.0028	8.31	8.31	0.034
Palmitate	0.0020	8.26	8.26	0.024
Stearate	0.0024	8.40	8.40	0.029
Temperature 35°				
Laurate	0.0049	8.02	8.02	0.061
Myristate	0.0037	8.42	8.42	0.044
Palmitate	0.0031	8.44	8.43	0.037
Stearate	0.0024	7.81	7.81	0.031
Temperature 50°				
Laurate	0.0052	8.58	8.57	0.061
Myristate	0.0038	8.52	8.52	0.045
Palmitate	0.0030	8.33	8.32	0.036
Stearate	0.0040	8.97	8.97	0.044

TABLE XI.

Solubility of Lithium Salts in Methyl Acetate.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 24.5°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0027	10.22	10.22	0.026
Myristate	0.0012	9.51	9.51	0.013
Palmitate	0.0015	9.87	9.87	0.015
Stearate	0.0012	9.69	9.69	0.012

TABLE XII.

Solubility of Lithium Salts in Acetone.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
	Temperature 15°			
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0234	7.83	7.81	0.300
Myristate	0.0322	7.82	7.79	0.413
Palmitate	0.0338	7.82	7.79	0.434
Stearate	0.0437	7.70	7.76	0.571
	Temperature 25°			
Laurate	0.0293	7.82	7.79	0.376
Myristate	0.0305	6.86	6.83	0.447
Palmitate	0.0396	7.83	7.79	0.508
Stearate	0.0510	7.27	7.22	0.706
	Temperature 35°			
Laurate	0.0329	7.67	7.64	0.430
Myristate	0.0385	7.71	7.67	0.502
Palmitate	0.0414	7.75	7.71	0.537
Stearate	0.0509	7.73	7.68	0.663

The magnesium salts were prepared by adding a slight excess of magnesium acetate to the warm alcoholic solutions of the fatty acids and the solution was set aside to cool. The laurate and myristate were recrystallized from hot alcohol while the palmitate and stearate were repeatedly washed with alcohol and dried.

TABLE XIII.

Solubility of Magnesium Salts in Water.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
	Temperature 15°			
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0010	9.85	9.85	0.010
Myristate	0.0006	9.87	9.87	0.006
Palmitate	0.0005	9.84	9.84	0.005
Stearate	0.0003	9.85	9.85	0.003
	Temperature 25°			
Laurate	0.0007	9.85	9.85	0.007
Myristate	0.0006	9.73	9.73	0.006
Palmitate	0.0008	9.84	9.84	0.008
Stearate	0.0004	9.86	9.86	0.004
	Temperature 35°			
Laurate	0.0010	9.83	9.83	0.010
Myristate	0.0007	9.83	9.83	0.007
Palmitate	0.0006	9.83	9.83	0.006
Stearate	0.0007	9.80	9.80	0.007
	Temperature 50°			
Laurate	0.0026	9.81	9.81	0.026
Myristate	0.0014	9.79	9.79	0.014
Palmitate	0.0009	9.82	9.82	0.009
Stearate	0.0008	9.79	9.79	0.008

TABLE XIV.

Solubility of Magnesium Salts in Ethyl Alcohol (Absolute).

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
	Temperature 15°			
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0404	7.82	7.78	0.519
Myristate	0.0123	7.79	7.78	0.158
Palmitate	0.0028	7.83	7.83	0.034
Stearate	0.0013	7.81	7.81	0.017
	Temperature 25°			
Laurate	0.0459	7.81	7.76	0.591
Myristate	0.0183	7.76	7.74	0.236
Palmitate	0.0045	7.77	7.76	0.058
Stearate	0.0018	7.77	7.77	0.023
	Temperature 35°			
Laurate	0.0630	7.76	7.70	0.805
Myristate	0.0287	7.75	7.72	0.373
Palmitate	0.0066	7.74	7.74	0.085
Stearate	0.0024	7.76	7.76	0.031
	Temperature 50°			
Laurate	0.0963	7.70	7.60	1.267
Myristate	0.0440	7.67	7 63	0.577
Palmitate	0.0116	7.67	7.66	0.151
Stearate	..	..	..	..

TABLE XV.

Solubility of Magnesium Salts in Methyl Alcohol.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 15°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0862	7.96	7.87	1.095
Myristate	0.0451	7.94	7.89	0.571
Palmitate	0.0180	7.93	7.92	0.227
Stearate	0.0066	7.93	7.92	0.084
Temperature 25°				
Laurate	0.0863	7.88	7.79	1.108
Myristate	0.0594	7.84	7.78	0.763
Palmitate	0.0264	7.88	7.85	0.336
Stearate	0.0078	7.84	7.84	0.100
Temperature 51.5°				
Palmitate	0.0384	7.72	7.68	0.500
Stearate	0.0128	7.72	7.71	0.166

TABLE XVI.

Solubility of Magnesium Salts in Ether.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 25°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0011	7.14	7.14	0.015
Myristate	0.0007	6.82	6.82	0.010
Palmitate	0.0003	7.49	7.49	0.004
Stearate	0.0002	7.47	7.47	0.003

TABLE XVII.

Solubility of Magnesium Salts in Ethyl Acetate.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
	Temperature 15°			
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0004	8.91	8.91	0.004
Myristate	0.0004	8.89	8.89	0.004
Palmitate	0.0004	8.91	8.91	0.004
Stearate	0.0004	8.70	8.70	0.004
	Temperature 35°			
Laurate	0.0010	8.74	8.74	0.011
Myristate	0.0009	8.76	8.76	0.010
Palmitate	0.0006	8.76	8.76	0.007
Stearate	0.0007	8.78	8.78	0.008
	Temperature 50°			
Laurate	0.0021	8.62	8.62	0.024
Myristate	0.0018	8.61	8.61	0.021
Palmitate	0.0011	8.60	8.60	0.013
Stearate	0.0010	8.63	8.63	0.011

TABLE XVIII.

Solubility of Magnesium Salts in Acetone.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
	Temperature 15°			
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0092	7.86	7.85	0.117
Myristate	0.0112	7.90	7.89	0.142
Palmitate	0.0131	7.89	7.89	0.166
Stearate	..	..	..	..
	Temperature 25°			
Laurate	0.0096	7.81	7.80	0.123
Myristate	0.0113	7.81	7.80	0.145
Palmitate	0.0125	7.81	7.80	0.160
Stearate	0.0150	7.85	7.83	0.191

TABLE XIX.

Solubility of Magnesium Salts in Amyl Alcohol.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 15°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0151	7.91	7.90	0.191
Myristate	0.0068	7.93	7.92	0.086
Palmitate	0.0034	7.99	7.98	0.043
Stearate	0.0011	7.89	7.89	0.014
Temperature 25°				
Laurate	0.0186	7.89	7.88	0.236
Myristate	0.0115	7.92	7.91	0.145
Palmitate	0.0052	7.94	7.93	0.066
Stearate	0.0014	7.95	7.95	0.018
Temperature 35°				
Laurate	0.1152	7.89	7.78	1.481
Myristate	0.0344	7.90	7.86	0.438
Palmitate	0.0082	7.85	7.84	0.104
Stearate	0.0031	7.88	7.88	0.039
Temperature 50°				
Laurate	0.3647	7.85	7.49	4.869
Myristate	0.1446	7.79	7.64	1.893
Palmitate	0.0205	7.81	7.79	0.263
Stearate	0.0082	7.82	7.81	0.105

TABLE XX.

Solubility of Magnesium Salts in Amyl Acetate.

	Salt.	Solution.	Solvent.	Salt in 100 gm solvent.
	Temperature 15°			
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0100	8.39	8.38	0.119
Myristate	0.0053	8.41	8.40	0.063
Palmitate	0.0033	8.43	8.42	0.039
Stearate	0.0025	8.42	8.42	0.029
	Temperature 25°			
Laurate	0.0135	8.31	8.30	0.162
Myristate	0.0061	8.35	8.34	0.073
Palmitate	0.0038	8.37	8.36	0.045
Stearate	0.0027	8.37	8.37	0.030
	Temperature 34.6°			
Laurate	0.0214	8.28	8.26	0.259
Myristate	0.0087	8.29	8.28	0.105
Palmitate	0.0047	8.30	8.30	0.057
Stearate	0.0038	8.29	8.29	0.046
	Temperature 50°			
Laurate	0.1553	8.16	8.01	1.939
Myristate	0.0491	8.16	8.11	0.605
Palmitate	0.0176	8.14	8.12	0.216
Stearate	0.0094	8.17	8.16	0.115

The barium salts were made by adding an alcoholic solution of $Ba(OH)_2$ to the warm alcoholic solutions of the acids and then washing the precipitates formed with hot alcohol. Even upon exercising the greatest care, a small amount of $BaCO_3$ was formed and precipitated from the alcoholic solution, but on account of the very slight solubility of the carbonate in the solvents used, no appreciable error in the solubility data is introduced from this source.

TABLE XXI.

Solubility of Barium Salts in Water.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 15.3°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0008	9.89	9.89	0.008
Myristate	0.0007	9.90	9.90	0.007
Palmitate	0.0004	9.90	9.90	0.004
Stearate	0.0004	9.89	9.89	0.004
Temperature 50°				
Laurate	0.0011	9.77	9.77	0.011
Myristate	0.0009	8.72	8.72	0.010
Palmitate	0.0007	9.84	9.84	0.007
Stearate	0.0006	9.86	9.86	0.006

TABLE XXII.

Solubility of Barium Salts in Ethyl Alcohol (Absolute).

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 16.5°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0008	7.82	7.82	0.010
Myristate	0.0007	7.84	7.84	0.009
Palmitate	0.0007	7.84	7.84	0.009
Stearate	0.0005	7.81	7.81	0.006
Temperature 25°				
Laurate	0.0008	7.78	7.78	0.010
Myristate	0.0009	7.79	7.79	0.011
Palmitate	0.0007	7.76	7.76	0.009
Stearate	0.0008	7.79	7.79	0.010
Temperature 35°				
Laurate	0.0010	7.72	7.72	0.013
Myristate	0.0010	7.73	7.73	0.013
Palmitate	0.0009	7.73	7.73	0.012
Stearate	0.0008	7.74	7.74	0.010
Temperature 50°				
Laurate	0.0005	7.60	7.60	0.007
Myristate	0.0003	7.67	7.67	0.004
Palmitate	0.0003	7.68	7.68	0.004
Stearate	0.0002	7.67	7.67	0.003

TABLE XXIII.

Solubility of Barium Salts in Methyl Alcohol.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 15°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0066	7.88	7.87	0.084
Myristate	0.0045	7.91	7.91	0.057
Palmitate	0.0036	7.91	7.90	0.045
Stearate	0.0033	7.89	7.89	0.042
Temperature 25°				
Laurate	0.0075	7.84	7.83	0.096
Myristate	0.0055	7.84	7.83	0.070
Palmitate	0.0040	7.86	7.86	0.051
Stearate	0.0038	7.85	7.84	0.049
Temperature 35°				
Laurate	0.0094	7.79	7.78	0.121
Myristate	0.0068	7.79	7.78	0.087
Palmitate	0.0058	7.80	7.80	0.074
Stearate	0.0050	7.82	7.82	0.066
Temperature 50.5°				
Laurate	0.0124	7.63	7.62	0.163
Myristate	0.0083	7.71	7.70	0.108
Palmitate	0.0068	7.71	7.70	0.088
Stearate	0.0060	7.76	7.75	0.077

TABLE XXIV.

Solubility of Barium Salts in Ether (Distilled over Sodium).

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 25°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0005	6.97	6.97	0.007
Myristate	0.0002	6.97	6.97	0.003
Palmitate	0.0001	7.00	7.00	0.001
Stearate	0.0001	6.92	6.92	0.001

TABLE XXV.

Solubility of Barium Salts in Amyl Alcohol.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 25°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0007	7.93	7.93	0.009
Myristate	0.0007	7.72	7.72	0.009
Palmitate	0.0006	7.94	7.94	0.008
Stearate	0.0006	7.90	7.90	0.007

The lead salts were made by adding calculated amounts of lead acetate, dissolved in a mixture of alcohol and water to alcoholic solutions of the fatty acids. The stearate and palmitate precipitates were washed by decantation with boiling alcohol and finally on the filter. The laurate was washed with cold alcohol and the myristate recrystallized from boiling alcohol. The salts were all amorphous powders except the laurate which comes down crystalline.

TABLE XXVI.

Solubility of Lead Salts in Water.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 35°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0009	9.85	9.85	0.009
Myristate	0.0005	9.85	9.85	0.005
Palmitate	0.0005	9.85	9.85	0.005
Stearate	0.0005	9.85	9.85	0.005
Temperature 50°				
Laurate	0.0007	9.82	9.82	0.007
Myristate	0.0006	9.81	9.81	0.006
Palmitate	0.0007	9.84	9.84	0.007
Stearate	0.0006	9.82	9.82	0.006

TABLE XXVII.

Solubility of Lead Salts in Ethyl Alcohol (Absolute).

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 25°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0007	7.80	7.80	0.009
Myristate	0.0003	7.80	7.80	0.004
Palmitate	0.0000	7.80	7.80	0.000
Stearate	0.0000	7.78	7.78	0.000
Temperature 35°				
Laurate	0.0025	7.74	7.74	0.032
Myristate	0.0003	7.73	7.73	0.004
Palmitate	0.0001	7.74	7.74	0.001
Stearate	0.0001	7.71	7.71	0.001
Temperature 50°				
Laurate	0.0202	7.67	7.64	0.264
Myristate	0.0040	7.66	7.66	0.052
Palmitate	0.0009	7.70	7.70	0.012
Stearate	0.0003	7.66	7.66	0.004

TABLE XXVIII.

Solubility of Lead Salts in Methyl Alcohol.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 15.5°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0048	7.91	7.90	0.061
Myristate	0.0044	7.87	7.87	0.056
Palmitate	0.0040	7.90	7.90	0.051
Stearate	0.0031	7.90	7.90	0.039
Temperature 25°				
Laurate	0.0075	7.85	7.84	0.096
Myristate	0.0061	7.85	7.85	0.078
Palmitate	0.0054	7.87	7.86	0.069
Stearate	0.0040	7.82	7.82	0.051
Temperature 35°				
Laurate	0.0088	7.79	7.78	0.113
Myristate	0.0064	7.81	7.81	0.082
Palmitate	0.0059	7.79	7.78	0.076
Stearate	0.0048	7.81	7.81	0.062
Temperature 50°				
Laurate	0.0216	7.73	7.71	0.280
Myristate	0.0092	7.70	7.69	0.119
Palmitate	0.0072	7.72	7.71	0.093
Stearate	0.0064	7.73	7.73	0.083

TABLE XXIX.

Solubility of Lead Salts in Ether (Distilled over Sodium).

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 14.5°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0008	7.04	7.04	0.010
Myristate	0.0009	6.96	6.96	0.013
Palmitate	0.0007	7.05	7.05	0.010
Stearate	0.0005	7.04	7.04	0.007

TABLE XXX.

Solubility of Lead Salts in Ethyl Acetate.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 14°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0015	8.89	8.89	0.017
Myristate	0.0009	8.93	8.93	0.010
Palmitate	0.0008	8.94	8.94	0.009
Stearate	0.0006	8.93	8.93	0.007
Temperature 35.5°				
Laurate	0.0031	8.75	8.75	0.035
Myristate	0.0013	8.76	8.76	0.015
Palmitate	0.0008	8.76	8.76	0.009
Stearate	0.0007	8.74	8.74	0.008
Temperature 50°				
Laurate	0.0182	8.68	8.66	0.210
Myristate	0.0067	8.67	8.67	0.077
Palmitate	0.0029	8.66	8.65	0.033
Stearate	0.0017	8.64	8.64	0.020

TABLE XXXI.

Solubility of Lead Salts in Benzene.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 15°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0010	8.71	8.71	0.011
Myristate	0.0009	8.71	8.71	0.010
Palmitate	0.0008	8.71	8.71	0.009
Stearate	0.0007	8.69	8.69	0.008

The silver salts were made by adding a calculated amount of ammoniacal silver nitrate dissolved in alcohol to alcoholic solutions of the acids. The white curdy precipitates resulting were extracted with hot 95 per cent alcohol, then washed on filters and dried.

TABLE XXXII.

Solubility of Silver Salts in Water.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 35°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	..	..	..	..
Myristate	0.0006	9.28	9.28	0.007
Palmitate	0.0004	9.85	9.85	0.004
Stearate	0.0004	9.85	9.85	0.004
Temperature 50°				
Laurate	..	..	..	..
Myristate	0.0007	9.83	9.83	0.007
Palmitate	0.0006	9.80	9.80	0.006
Stearate	0.0004	9.82	9.82	0.004

TABLE XXXIII.

Solubility of Silver Salts in Ethyl Alcohol (Absolute).

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
Temperature 25°				
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0007	7.78	7.78	0.009
Myristate	0.0006	7.76	7.76	0.008
Palmitate	0.0005	7.77	7.77	0.007
Stearate	0.0003	7.77	7.77	0.007
Temperature 50°				
Laurate	0.0007	7.68	7.68	0.009
Myristate	0.0006	7.67	7.67	0.008
Palmitate	0.0005	7.67	7.67	0.007
Stearate	0.0005	7.67	7.67	0.007

TABLE XXXIV.

Solubility of Silver Salts in Methyl Alcohol.

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
	Temperature 15°			
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0058	7.88	7.87	0.074
Myristate	0.0050	7.89	7.89	0.063
Palmitate	0.0048	7.91	7.90	0.060
Stearate	0.0040	7.91	7.91	0.051
	Temperature 25°			
Laurate	0.0056	7.84	7.84	0.072
Myristate	0.0053	7.87	7.86	0.067
Palmitate	0.0046	7.88	7.88	0.059
Stearate	0.0041	7.87	7.87	0.052
	Temperature 35°			
Laurate	0.0061	7.78	7.77	0.078
Myristate	0.0055	7.80	7.80	0.071
Palmitate	0.0048	7.79	7.79	0.062
Stearate	0.0043	7.80	7.79	0.055
	Temperature 50°			
Laurate	0.0064	7.70	7.70	0.083
Myristate	0.0056	7.72	7.71	0.073
Palmitate	0.0051	7.71	7.71	0.066
Stearate	0.0046	7.71	7.71	0.060

TABLE XXXV.

Solubility of Silver Salts in Ether (Distilled over Sodium).

	Salt.	Solution.	Solvent.	Salt in 100 gm. solvent.
	Temperature 15°			
	gm.	*gm.*	*gm.*	*gm.*
Laurate	0.0007	6.99	6.99	0.010
Myristate	0.0006	7.02	7.02	0.009
Palmitate	0.0006	6.75	6.75	0.009
Stearate	0.0005	7.04	7.04	0.007

CONCLUSIONS.

From the above tables it is seen that the solubility of all the salts of the four fatty acids in the various solvents tried is only slight, but that considerable differences are found not only among the several salts in the same solvent, but also for the same salts in the different solvents.

The solubility in any case rarely exceeds 1 per cent, but was found to vary between 6 per cent and virtual insolubility. Methyl alcohol was found to be the best general solvent for this class of substances.

The lithium salts were found to be about three times as soluble in methyl alcohol and acetone as the magnesium salts, while the latter are more soluble in ethyl alcohol than the former.

It is also seen that the lithium salts are a great deal more soluble in water than the magnesium salts, but the difference is not a constant ratio for the different temperatures.

The beryllium salts of the fatty acids were made, but contrary to expectations we found that the basic salts, rather than the normal, were formed.

THE SEPARATION OF LAURIC AND MYRISTIC ACIDS FROM EACH OTHER AND FROM MIXTURES OF OTHER FATTY ACIDS.*

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With the preceding paper[1] as a basis, the authors have succeeded in working out what appear to be practical methods for the isolation of lauric and myristic acids when present in mixtures of the higher fatty acids.

By way of introduction it should be said that, in general, all fatty acids occurring in nature may be classified in three main groups: the saturated, unsaturated, and hydroxy acids.[2] The acids of these three major groups may be subdivided into subgroups, either with reference to properties or molecular structure. The saturated fatty acids are best adapted to a property classification, while the acids of the other two groups may be classified with respect to their molecular structure. According to this grouping the hydroxy acids are subdivided into mono-, di-, tri-, etc., hydroxy acids, and the unsaturated acids into those having a double bond, such as oleic acid; a triple bond, such as stearolic acid; and a double and triple bond, such as ricinstearolic acid. The property relations of these subgroups have not been worked out satisfactorily.

The differential grouping of the saturated acids, with which this paper has to deal, is based on property relations, which may be formulated as follows: *Group I*, all the lower members up to and including caprylic acid. These are all capable of distillation at

* This investigation was carried out at the Nevada Agricultural Experiment Station with funds obtained under the Adams Act.

[1] Jacobson, C. A., and Holmes, A., *J. Biol. Chem.*, 1916, xxv, 29.

[2] This is on the assumption that the amino and other substituted fatty acids are excluded.

atmospheric pressure without decomposition, and are more or less easily soluble in water. The Reichert-Meissl number is a fairly accurate criterion for the quantity of Group I acids in a given sample. *Group 2*, all the acids between and including capric and stearic acids. These are more or less soluble in cold and hot 90 per cent alcohol; but a regular gradation in solubility is found between the upper and lower members of the group. They cannot be distilled at ordinary pressures without decomposition, although they are volatile with steam. *Group 3*, all acids above stearic acid. They are practically insoluble in cold 90 per cent alcohol, and in general not volatile with steam.

For the separation of the fatty acids into these groups, as well as for the isolation of individual members of each group, a large number of proposed methods are to be found in the literature. A discussion of all those not bearing more or less directly upon the separations in Group 2 of the saturated acids, will be omitted.

This grouping is arbitrary, and the authors have been unable to find any property that would exactly fit one group to the exclusion of those adjacent. To illustrate, if we were to distill a mixture of the saturated fatty acids of Groups 1 and 2, we should find that certain amounts of some of the so called soluble volatile fatty acids of Group 1 would remain behind, and certain fractions of a few of the acids of Group 2 would be found in the distillate.

The Reichert-Wollny process[3] is perhaps the best for determining the acids of Group 1, and the Gusserow-Varrentrapp, lead-salt-ether method[4] the best for separating the unsaturated acids from the acids of this group. Various other methods for these separations have been proposed, but certain undesirable features accompany all of them.

The quantitative separation of the acids of Group 2 from those of Group 3 of this series is also fraught with considerable difficulty, but the method of dissolving in boiling 90 per cent alcohol and cooling to room temperature, using the proper concentration of the solution, would appear to be the best at present.

For the separation of individual members of Group 2, to which lauric and myristic acids belong, various methods have been proposed, such as the fractional precipitation of their salts, the frac-

[3] Reichert, E., and Wollny, R., *Analyst*, 1900, xxv, 309.
[4] Varrentrapp, F., *Ann. Chem.*, 1840, xxxv, 197.

tional distillation *in vacuo* of the acids, and of their methyl and ethyl esters.

Gsell[5] worked out a method for separating capric acid from the other members of this group by making anhydrides of the acids in ethereal solution, dissolving in pyridine, and pouring this solution into water. He says the mixed anhydride of capric acid will remain dissolved, while the normal anhydrides of the other acids will be precipitated. Lewkowitsch[6] attempted to make anhydrides of these acids, but from his results he concluded that "the acid values found for the products of the interaction of acetic anhydride and fatty acids lose every quantitative meaning."

For the separation of stearic and palmitic acids, Kreis and Hafner[7] have worked out a modification of Hehner and Mitchell's method,[8] but it is not found to be entirely satisfactory.

The authors tested out experimentally two well known methods for the separation of the fatty acids in this group, but the results were far from satisfactory. The methods tried were Facchini and Dorta's,[9] and Partheil and Ferié's.[10] The former method depends upon the difference in solubility of the potassium soaps in acetone at different temperatures, while the latter depends upon the difference in solubility of the potassium soaps in 50 per cent and 100 per cent alcohol.

Following are the results from the latter method: 0.500 gm. of each of lauric, myristic, palmitic, and stearic acids were dissolved in alcohol, neutralized with 0.2 N alcoholic potash, the volume was made up to 200 cc. and sufficient water added to bring the concentration of alcohol to 50 per cent by volume. The acids were precipitated by adding 10 per cent lithium acetate in 50 per cent alcoholic solution. The mixture was then heated until all the salts passed into solution, and set aside to cool. The crystallized salts thus obtained were filtered off, giving Precipitate I in Table I, and the filtrate was treated with 10 per cent aqueous lead acetate, filtered, washed, and the fatty acids were liberated with hydrochloric acid.

[5] Gsell, J., *Chem. Zeit.*, 1907, xxxi, 100.
[6] Lewkowitsch, J., *J. Chem. Soc.*, 1890, Proc. 92.
[7] Kreis, H., and Hafner, A., *Ber. chem. Ges.*, 1903, xxxvi, 2766.
[8] Hehner, O., and Mitchell, C. A., *Analyst*, 1896, xxi, 320.
[9] Facchini, S., and Dorta, W., *Chem. Rev. Fett.-u. Harz-Ind.*, 1912, xix, 77.
[10] Partheil, A., and Ferié, F., *Arch. Pharm.*, 1903, ccxli. 545.

The acids thus obtained constitute Precipitate II. Precipitate I was boiled with absolute alcohol, the solution allowed to cool, the precipitate formed was filtered and washed, which constitutes Precipitate III. Precipitate IV was obtained by evaporating the filtrate from Precipitate III to dryness.

The fatty acids of Precipitates II and III were liberated from their lithium soaps by hydrochloric acid, washed, dried, and weighed. The neutralization values of the acids from these pre-

TABLE I.

	a.	b.	c.
Precipitate I.			
Yield { Gm	1.300	0.888	1.092
Yield { Per cent	63.4 (With 100 cc. absolute alcohol.)	43.3 (With 150 cc. absolute alcohol.)	53.3 (With 150 cc. absolute alcohol.)
Precipitate II.			
Yield	0.603 gm.	0.996 gm.	0.765 gm.
Neutralization value	266	252.5	258
Per cent lauric acid	60	19	35
" " myristic "	40	81	65
Precipitate III.			
Yield	0.940 gm.	0.716 gm.	0.913 gm.
Neutralization value	214	207.9	209.1
Per cent stearic acid	29	52	46
" " palmitic "	71	48	54
Precipitate IV.			
Yield	0.339 gm.	0.156 gm.	0.149 gm.
Neutralization value	232	219	236
Per cent palmitic acid	52	100	37
" " myristic "	48	—	63

cipitates were obtained and will also be found in Table I. It was learned that the amount of lithium acetate added influenced the character of the soap mixtures in the different precipitates. Under Column a will be found the results obtained when a large excess of lithium acetate was added; under Column b, when 10 cc. of a 10 per cent solution were added; and under Column c, when 20 cc. of the acetate were used.

The results here obtained indicate that no quantitative separa-

tion can be obtained by this method, but that a small amount of pure palmitic acid was isolated in Precipitate IV by using 10 cc. of lithium acetate solution and 150 cc. of absolute alcohol as the solvent. The yield, however, was only 31.2 per cent in this case. The results from the method of Facchini and Dorta were no more encouraging.

From an inspection of the solubility data of the salts of lauric, myristic, palmitic, and stearic acids given in the preceding paper,[1] we learn that only two possible methods for separations appear. They are as follows: First, it is shown that lithium laurate is about five times as soluble in water as lithium myristate, the next higher salt, at the four temperatures investigated, and this suggests the possibility of extracting the laurate from a mixture of lithium salts with water or the precipitation of the three salts from water, retaining the laurate in solution. Second, it is seen that the solubility of magnesium myristate is about five times as great as magnesium palmitate in absolute alcohol at 15° and 25°, suggesting the possibility of separating the myristate from the two higher salts after the lauric acid has been removed as the lithium salt by the foregoing method.

To test these possibilities, the following experiments were carried out.

Experiment 1.—0.5 gm. of each of the four fatty acids were weighed out, mixed, and dissolved in strong alcohol, and the solution was neutralized with 0.2 N alcoholic potassium hydroxide. The mixed acids had a neutralization value of 231.3; calculated value for the mixture: 235.8. The alcohol was evaporated off and the soaps were taken up in 300 cc. water, after which a calculated amount of lithium acetate was added to make the lithium salts. The mixture was boiled, cooled, and filtered. The precipitate thus obtained weighed 1.65 gm., or was a yield of 80 per cent. The filtrate was evaporated to three-fourths of its volume, cooled, filtered, and the fatty acid liberated with dilute hydrochloric acid. The acid, weighing 0.155 gm., had a neutralization value of 271, whereas the corresponding value for lauric acid is 280.5.

These results suggested the possibility of a separation by this method upon varying the conditions somewhat.

Experiment 2.—Again, a mixture of 0.5 gm. of each of the four acids was converted into potassium soaps and, after evaporating off the alcohol, was taken up in 400 cc. of water and precipitated with a slight excess of lithium acetate solution. In this case the precipitated lithium salts

weighed 1.562 gm., equivalent to 1.527 gm. of acid mixture, which was 76.3 per cent of the original acid mixture, whereas 75 per cent should have been obtained if the separation had been perfect.

The filtrate was concentrated to one-half its volume, cooled, and filtered, yielding a precipitate weighing 0.208 gm., and the acid obtained from this precipitate yielded a neutralization value of 278.3, and had a melting point of 43.2°, whereas the acid obtained by evaporating the filtrate to dryness gave a neutralization value of 271.2 and the same melting point. Calculated for lauric acid: 280.5 and 43.6° respectively.

The water-insoluble salts from this separation yielded an acid mixture having a neutralization value of 217.5, whereas a mixture of equal quantities of myristic, palmitic, and stearic acids gives a neutralization value of 220.9.

It was next tried to separate myristic acid from a mixture of the three higher acids of this group by means of the difference in the solubility of their magnesium salts in absolute alcohol, but the experiments resulted unsuccessfully, doubtless because of the influence of one salt exerted upon the solubility of the others, in this solvent. Instead of absolute alcohol, 50 per cent alcohol was used as the medium in which the precipitation of the magnesium salts was carried out, according to the conditions given in the following experiment.

Experiment 3.—0.5 gm. each of magnesium myristate, palmitate, and stearate were digested with 60 cc. of 50 per cent alcohol for 2 hours at 60°, then cooled, and filtered. The fatty acids were liberated from the filtrate, washed, dried, and weighed, giving 0.270 gm. with a melting point of 48.6° and a neutralization value of 243. Since the yield was only about one-half of what it should be, the insoluble salts were again extracted with alcohol in the same manner, yielding an insoluble residue weighing 0.928 gm. This time the acids were liberated from the insoluble residue and after being recrystallized from 60 per cent alcohol showed a melting point of 56.5° and a neutralization value of 209.3. Calculated for a mixture of equal parts of stearic and palmitic acids: 56.4° and 208.3 respectively.

The acids obtained from this insoluble residue must therefore be a mixture of equal quantities of stearic and palmitic acids, showing that the myristic acid had been almost completely removed.

A subsequent experiment using 100 cc. of 50 per cent alcohol did not yield as good results as the foregoing but a fairly good separation was effected.

During the course of the investigation it was noticed that after

neutralizing an alcoholic solution of these fatty acids with alcoholic potassium hydroxide, and allowing the solution to stand over night, a white crystalline and a gelatinous precipitate appeared in the flask the next morning. These two were so different in properties that they could be separated by decantation, but upon investigation it was found that both forms yielded practically the same fatty acid mixture. Whether they represented allotropic modifications or simply crystalline and colloidal forms of the potassium salts of the fatty acid mixture was left undetermined.

CONCLUSION.

In the present paper the authors have presented a method for the separation of lauric acid when present in a mixture of myristic, palmitic, and stearic acids, and a method for the separation of myristic acid from a mixture in which it occurs, together with palmitic and stearic acids. These methods are based upon the differences of the solubility of their lithium and magnesium salts in water and 50 per cent alcohol.

CHANGES IN THE H^+ AND OH^- CONCENTRATION WHICH TAKE PLACE IN THE FORMATION OF CERTAIN PROTEIN COMPOUNDS.

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It has been known for some time that certain proteins combine with other proteins to form compound proteins. Kossel[1] states that the protamines in weakly alkaline solutions combine with proteins to form compounds which precipitate under proper conditions. Kutscher,[2] working with albumoses obtained from Witte's peptone prepared compounds not only with protamine but also with various other proteins. While studying the properties of the histones, Bang[3] was able to confirm the observations of Kutscher and further showed that various members of this group combine with proteins to form compounds. Kossel and Kutscher[4] surmised that histones (on account of the high content of basic substances) might be a combination of protamine with other proteins but later Kossel and Pringle[5] showed that this view was incorrect since in the first step in the peptic digestion of histones, histopeptone is formed, whereas protamine-protein combinations are split into protamine and protein. Hunter[6] prepared a number of compounds of protamine with various proteins, such combinations as clupein-casein, clupein-gelatin, and clupein-edestin being obtained. He further determined the proportion of the two substances which unite to form the compound protein. Gay and Robertson,[7] studying the antigenic properties of

[1] Kossel, A., *Deutsch. med. Woch.*, 1894, xx, 147.
[2] Kutscher, F., *Z. physiol. Chem.*, 1897, xxiii, 115.
[3] Bang, I., *Z. physiol. Chem.*, 1899, xxvii, 463.
[4] Kossel, A., and Kutscher, F., *Z. physiol. Chem.*, 1900–01, xxxi, 165.
[5] Kossel, A., and Pringle, H., *Z. physiol. Chem.*, 1906, xlix, 301.
[6] Hunter, A., *Z. physiol. Chem.*, 1907, liii, 526.
[7] Gay, F. P., and Robertson, T. B., *J. Exp. Med.*, 1912, xvi, 479.

compound proteins, prepared the compound protamine (salmine) caseinate. Robertson,[8] unaware of the work of Bang, surmised from the possible relation of the histones to the protamines that globin might also unite with other proteins to form compounds. He prepared globin caseinate and determined its refractive index in 0.1 N KOH solution. Later Gay and Robertson[9] studied its antigenic properties.

It will be noted that in the work recorded precipitation of the compound took place in neutral or alkaline solutions, although Kossel[1] states that a nuclein may be formed in acid solution from the combination of nucleic acid and protein. Altmann[10] had previously observed that proteins could combine in acid solution and Milroy[11] prepared a number of protein-nucleic acid compounds. Apparently no observations have been made to determine the true acidity (or alkalinity) of the protein solutions used or of the mixture when precipitation of the compound took place. The conditions for precipitation must be such that salting out of either of the proteins by inorganic salts formed during the reaction must not take place and the acidity of the solution must be such that the compound protein, if formed, will not be dissolved.

In the present work use of the gas chain has been made to follow the change of acidity (or alkalinity) of the solution during the precipitation of a compound protein to distinguish, if possible, salting out of either protein from true compound precipitation. Determination of the exact hydrogen ion concentration serves as a criterion for the duplication of any work in the preparation of compound proteins, since it appears that the exact acidity (or alkalinity) is a very important factor in the preparation of such compounds.

Use of the hydrogen electrode has been made by a large number of observers. Böttger[12] made use of the gas chain to determine the neutral point in the titration of acids and bases. The use of the hydrogen elec-

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[8] Robertson, T. B., *J. Biol. Chem.*, 1912–13, xiii, 499.

[9] Gay and Robertson, *J. Exp. Med.*, 1913, xvii, 535.

[10] Altmann, R., *Arch. Anat. u. Physiol., Physiol. Abt.*, 1889, 524.

[11] Milroy, T. H., *Z. physiol. Chem.*, 1896–97, xxii, 307.

[12] Böttger, W., *Z. physik. Chem.*, 1897, xxiv, 253.

trode for such purposes has been emphasized by Hildebrand,[13] who points out its many applications in the analytical field. Salm,[14] determining the H^+ concentrations in various phosphate mixtures, was able to prepare a scale of indicators for the colorimetric estimation of H^+ and OH^- concentrations. Schmidt and Finger[15] showed that solutions of definite H^+ and OH^- concentrations may be made by using mixtures of various borates. They also point out that from the shape of the titration curve the existence of a compound in solution may be determined. Thus they confirmed observations made by various other methods by Noyes and Whitney,[16] Kahlenberg and Schreiner,[17] and Shelton[18] that a compound NaH_2BO_3, or the anhydride $NaBO_2$, or the corresponding ions H_2BO_3 and BO_2 exist in solution. In the field of protein chemistry much use has also been made of the hydrogen electrode. Thus Robertson[19] used it to determine the dissociation of serum globulin at varying hydrogen ion concentrations, and the dissociation of potassium caseinate[20] and ovomucoid[21] at varying alkalinities. Robertson and Schmidt[22] were able to follow the change of alkalinity which took place during the progress of tryptic digestions of certain proteins. The many uses of the hydrogen electrode are set forth in a monograph by Michaelis.[23]

The plan of these experiments was to determine the H^+ or OH^- concentration in a solution of protein "*a*". Definite amounts of either a solution of protein "*b*" or an inorganic salt used to precipitate protein "*a*" from its solution, were then added to the solution of protein "*a*". The H^+ concentration was then determined in the solution of protein "*a*" after each addition of the solution of protein "*b*". A titration curve can thus be plotted using cc. of solution "*b*" as ordinates and the resultant H^+ or OH^- concentrations as abscissæ. In this way a titration curve is obtained similar to those described by Böttger,[12] Schmidt and Finger,[15] and Hildebrand.[13]

[13] Hildebrand, J. H., *J. Am. Chem. Soc.*, 1913, xxxv, 847.

[14] Salm, E., *Z. Electrochem.*, 1904, x, 341; *Z. physik. Chem.*, 1907, lvii, 471.

[15] Schmidt, C. L. A., and Finger, C. P., *J. phys. Chem.*, 1908, xii, 406. See also Hildebrand, J. H., and Bowers, W. G., *J. Am. Chem. Soc.*, 1916, xxxviii, 785.

[16] Noyes, A. A., and Whitney, W. R., *Z. physik. Chem.*, 1894, xv, 694.

[17] Kahlenberg, L., and Schreiner, O., *Z. physik. Chem.*, 1896, xx, 547.

[18] Shelton, H. S., *Z. physik. Chem.*, 1903, xliii, 494.

[19] Robertson, *J. phys. Chem.*, 1907, xi, 437.

[20] Robertson, *J. phys. Chem.*, 1910, xiv, 528.

[21] Robertson, *J. phys. Chem.*, 1910, xiv, 709.

[22] Robertson, and Schmidt, *J. Biol. Chem.*, 1908, v, 31.

[23] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.

The method used to determine the H^+ concentrations was essentially the same as used by Robertson[20,21] and Robertson and Schmidt,[22] and described by Schmidt and Finger[15] and Robertson.[24] Hydrogen was generated by the electrolysis of a 6 per cent by volume solution of concentrated H_2SO_4 and passed over heated platinized asbestos to rid it of any oxygen or oxygen compounds. The hydrogen then passed through the solution at two points, through a nozzle at the bottom of the cell and

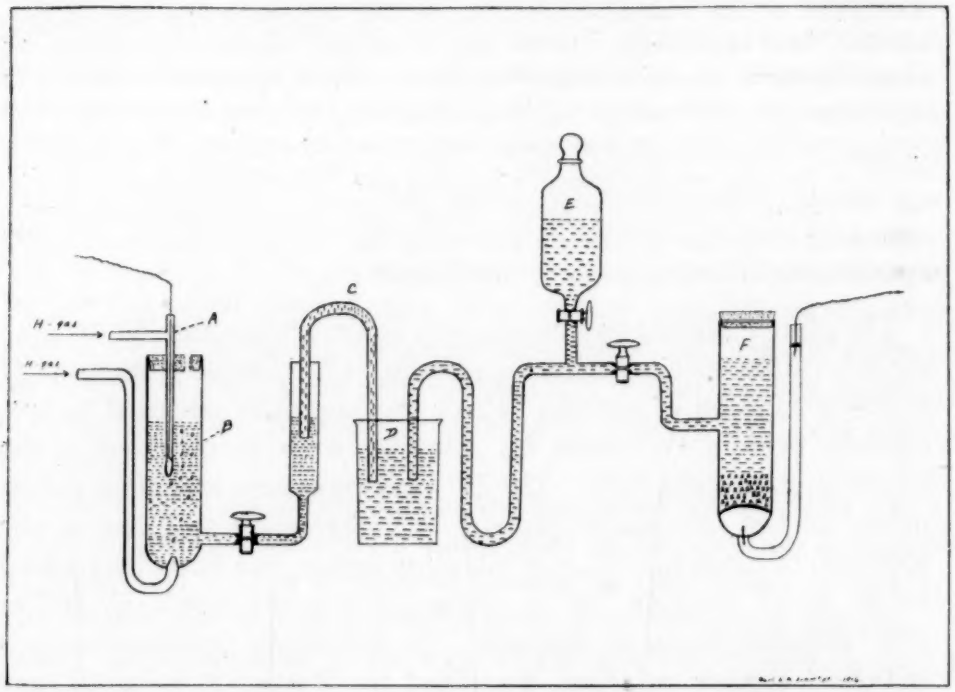

FIG. 1. A, platinum gauze electrode; B, titration cell; C, agar tube saturated with KCl; D, beaker with 0.1 N KCl; E, separatory funnel with 0.1 N KCl; F, 0.1 N KCl—HgCl—Hg—electrode.

through a Cottrell gauze electrode.[15, 25] In this way the three phases, platinum, hydrogen, and solution were brought into intimate contact. A calomel electrode,[26] using 0.1 N KCl, was used as the other extremity of the chain. The apparatus is shown in Fig. 1. A is the gauze electrode dipping into the solu-

[24] Robertson, Die physikalische Chemie der Proteine, Dresden, 1912, 417.

[25] Robertson, *J. phys. Chem.*, 1907, xi, 442. Loomis, N. E., Dissertation, Johns Hopkins Univ., 1911, 22. Loomis, N. E., and Acree, S. F., *Am. Chem. J.*, 1911, xlvi, 602.

[26] Richards, T. W., *Z. physik. Chem.*, 1897, xxiv, 39.

tion contained in cell B, D a beaker containing 0.1 N KCl, and F the calomel electrode. Connection with cell B is made by means of an agar tube saturated with KCl which eliminates contact potential.[27, 28] To eliminate contamination of the solution in cell B, the stop-cock connecting the side arm with the cell was kept closed except during the measurement of the potential and likewise the agar tube was dipped into the solution in the side arm only while readings were being taken. Through a hole in the stopper of cell B any amount of a solution "*b*" could be introduced. Measurement of the potential was made on a 100 cm. bridge using a sensitive Leeds and Northrup galvanometer[29] as zero instrument. Three Edison-Lalande cells connected in series furnished current through the potentiometer wire and the E.M.F. was checked against a Weston cell after each reading. The protein solutions "*a*" and "*b*" as well as the gauze electrode were separately saturated with hydrogen before bringing them together, the necessity for this having been shown by Robertson[20] and by Desha and Acree.[30] Hydrogen was allowed to bubble through the solution from 45 to 60 minutes before determining the E.M.F., and this was likewise done after each addition of solution "*b*". All determinations were made at room temperature. To prevent foaming a few drops of octyl alcohol were floated on the surface of the solution in B after the introduction of the electrode. The H^+ and OH^- concentrations corresponding to the E.M.F.'s were taken from tables previously calculated by Schmidt.[31]

Since it is not feasible to determine H^+ or OH^- concentrations in ammoniacal solutions by means of the gas chain, the experi-

[27] Bjerrum, N., *Z. physik. Chem.*, 1905, liii, 428. Loomis, N. E., Dissertation, Johns Hopkins Univ., 1911, 34. Loomis, N. E., and Acree, S. F., *Am. Chem. J.*, 1911, xlvi, 585. Clark, F. W., Myers, C. N., and Acree, S. F., *J. phys. Chem.*, 1916, xx, 241. Bjerrum, *Z. Electrochem.*, 1911, xvii, 389. See also Cumming, A. C., and Abegg, R., *Z. Electrochem.*, 1907, xiii, 17.

[28] This may not be strictly true, but since we are not concerned with absolute values but merely with changes in voltage this factor may be neglected.

[29] Kindly loaned by the Physics Department.

[30] Desha, L. J., and Acree, S. F., *Am. Chem. J.*, 1911, xlvi, 638.

[31] Schmidt, C. L. A., *Univ. California Publications, Physiology*, 1905–10, iii, 101.

ments were made with compounds of globin. As stated by Bang[3] and Robertson,[8] precipitation of the compound protein takes place in slight excess of NaOH or KOH. Globin was prepared according to a modification of the method described by Robertson.[32] Two preparations were made, one precipitated by NH_4Cl from an ammoniacal solution and the other by alcohol and ether from HCl solution. These correspond to Preparations I and II described by Robertson. Preparation II is soluble in water without addition of acid or alkali. The casein employed was Eimer and Amend's ("*nach Hammarsten*") which had been further purified according to Robertson.[33] Deuteroalbumose was made from Witte's peptone as described by Kutscher.[2] The nucleic acid was a preparation extracted by Dr. A. E. Taylor from the sperm of the Pacific Coast salmon (probably according to the method of Altmann).[10] The bile salts were prepared from ox bile according to the method of Plattner.[34]

The following is a tabular representation of the results obtained in the experiment. In the first column are given the volumes of the titrating solution "*b*" which were added to a given amount of solution "*a*". The second column shows the E.M.F. determined after the establishment of equilibrium. The H^+ and OH^- concentrations corresponding to the E.M.F.'s are given in the next two columns. In the last column are given the calculated possible errors in determining the H^+ concentration on the assumption that the determination of the E.M.F.'s was accurate to a millivolt.

Curves showing either the change in the H^+ or the OH^- concentration which took place during the titration as determined by experiment are plotted and given below. These will be referred to as titration curves. The first experiments were carried out to determine what effect was produced on the H^+ concentration by simple dilution and by salting out with those salts which might be formed as intermediate products of the reaction; so that, if possible, a distinction between salting out and true protein com-

[32] Robertson, *J. Biol. Chem.*, 1912–13, xiii, 455.

[33] Robertson, *J. Biol. Chem.*, 1906–07, ii, 317; *J. phys. Chem.*, 1910, xiv, 534.

[34] Plattner, E. A., *J. prakt. Chem.*, 1847, xl, 129.

pound formation might be made. The results are given in Tables I, II, and III and shown graphically in Fig. 2. The change in H^+ concentration is a direct function of the titrating solution and the curves are straight lines, despite the fact that with both NH_4Cl and KCl a precipitate is obtained. In these instances we apparently have either a purely physical phenomenon or a chemical one which involves no sudden change in the H^+ concentration such as to give a break in the curve. The precipitation of proteins by inorganic salts has been studied extensively

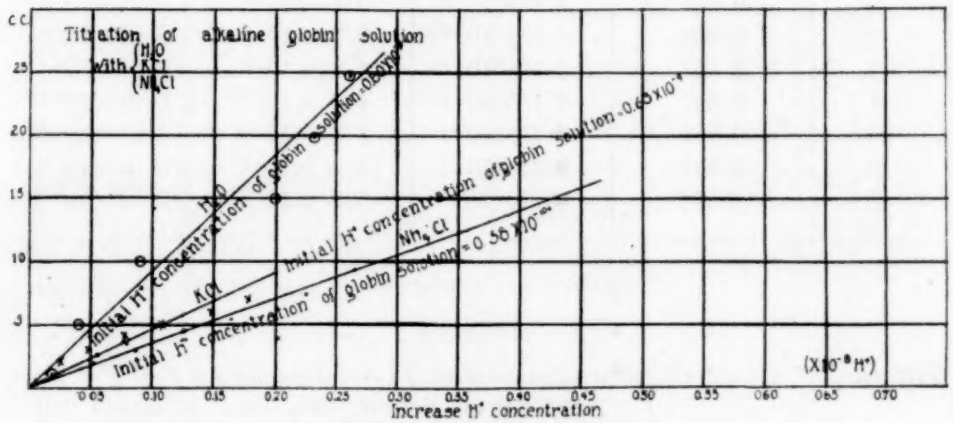

FIG. 2.

TABLE I.

Titration of Alkaline Globin (Preparation I) Solution with Water.

Concentration of globin, 0.5 per cent. 0.25 gm. globin dissolved by 8 cc. 0.1 N KOH, then titrated back with 7 cc. 0.1 N HCl. 50 cc. globin solution titrated. No precipitate produced.

H_2O added.	E.M.F.	H^+	OH^-	Possible error in H^+
cc.	*volts*			
0	0.861	0.80×10^{-9}	0.80×10^{-5}	$\pm0.06\times10^{-9}$
1	0.856	0.98×10^{-9}	0.65×10^{-5}	0.08×10^{-9}
5	0.850	0.12×10^{-8}	5.3×10^{-6}	0.01×10^{-8}
10	0.842	0.17×10^{-8}	3.8×10^{-6}	0.01×10^{-8}
15	0.830	0.28×10^{-8}	2.3×10^{-6}	0.03×10^{-8}
20	0.827	0.31×10^{-8}	2.1×10^{-6}	0.02×10^{-8}
25	0.825	0.34×10^{-8}	1.9×10^{-6}	0.03×10^{-8}

TABLE II.

Titration of Alkaline Globin (Preparation I) Solution with a Saturated Solution of KCl.

Concentration of globin, 0.5 per cent. 0.25 gm. globin dissolved by 8 cc. 0.1 N KOH, then titrated back with 7 cc. 0.1 N HCl. 50 cc. globin solution titrated. Precipitate produced.

KCl solution added.	E.M.F.	H^+	OH^-	Possible error in H^+
cc.	*volts*			
0	0.867	0.63×10^{-9}	1.0×10^{-5}	$\pm0.05\times10^{-9}$
1	0.859	0.87×10^{-9}	0.74×10^{-5}	0.07×10^{-9}
2	0.859	0.87×10^{-9}	0.74×10^{-5}	0.07×10^{-9}
3	0.853	0.11×10^{-8}	5.8×10^{-6}	0.01×10^{-8}
4	0.847	0.14×10^{-8}	4.6×10^{-6}	0.01×10^{-8}
5	0.842	0.17×10^{-8}	3.8×10^{-6}	0.01×10^{-8}
6	0.837	0.21×10^{-8}	3.1×10^{-6}	0.02×10^{-8}
7	0.834	0.24×10^{-8}	2.7×10^{-6}	0.02×10^{-8}

TABLE III.

Titration of Alkaline Globin (Preparation I) Solution with a 0.25 Per Cent Solution of NH_4Cl.

Concentration of globin, 0.5 per cent. 0.25 gm. globin dissolved by 8 cc. 0.1 N KOH, then titrated back with 7 cc. 0.1 N HCl. 50 cc. globin solution titrated. Precipitate produced.

NH_4Cl solution added.	E.M.F.	H^+	OH^-	Possible error in H^+
cc.	*volts*			
0	0.927	0.58×10^{-10}	1.1×10^{-4}	$\pm0.05\times10^{-10}$
0.5	0.911	0.11×10^{-9}	5.8×10^{-5}	0.01×10^{-9}
1.0	0.893	0.22×10^{-9}	2.9×10^{-5}	0.01×10^{-9}
1.5	0.874	0.48×10^{-9}	1.3×10^{-5}	0.04×10^{-9}
2.0	0.869	0.58×10^{-9}	1.1×10^{-5}	0.04×10^{-9}
2.5	0.868	0.61×10^{-9}	1.1×10^{-5}	0.05×10^{-9}
3.0	0.857	0.94×10^{-9}	0.68×10^{-5}	0.07×10^{-9}
3.5	0.859	0.87×10^{-9}	0.74×10^{-5}	0.07×10^{-9}
4.5	0.848	0.13×10^{-8}	4.9×10^{-6}	0.01×10^{-8}
5.5	0.843	0.17×10^{-8}	3.9×10^{-6}	0.02×10^{-8}
7.5	0.835	0.23×10^{-8}	2.8×10^{-6}	0.02×10^{-8}
9.5	0.831	0.27×10^{-8}	2.4×10^{-6}	0.03×10^{-8}
14.5	0.819	0.43×10^{-8}	1.5×10^{-6}	0.03×10^{-8}

by Hardy,[35] Pauli,[36] Hofmeister,[37] Galeotti,[38] and many others, and is discussed at length by Robertson.[39]

The titration of globin in acid and alkali solutions by alkali and acid respectively was next studied. For this purpose Preparation I, dissolved by a small amount of alkali, and Preparation II, which is water-soluble and acid in reaction, were used. During

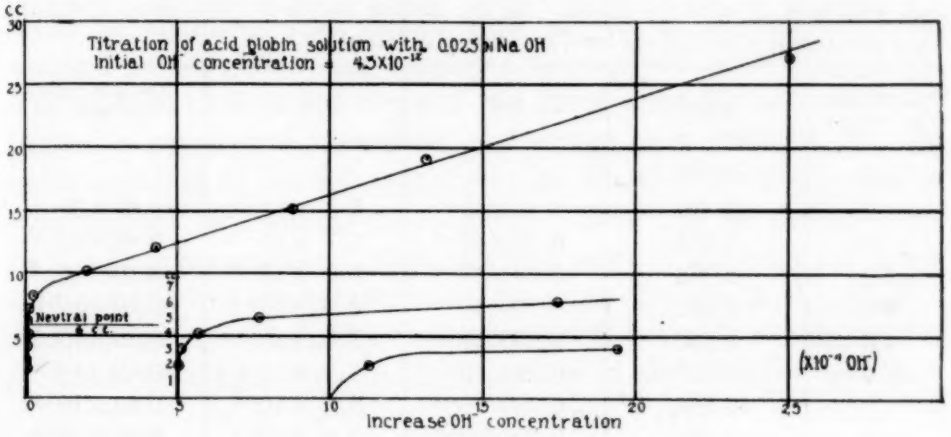
Titration of acid globin solution with 0.025 N Na OH
Initial OH' concentration = 4.5X10⁻¹²
Neutral point
8 cc
(X10⁻⁴ OH')
Increase OH' concentration

FIG. 3.

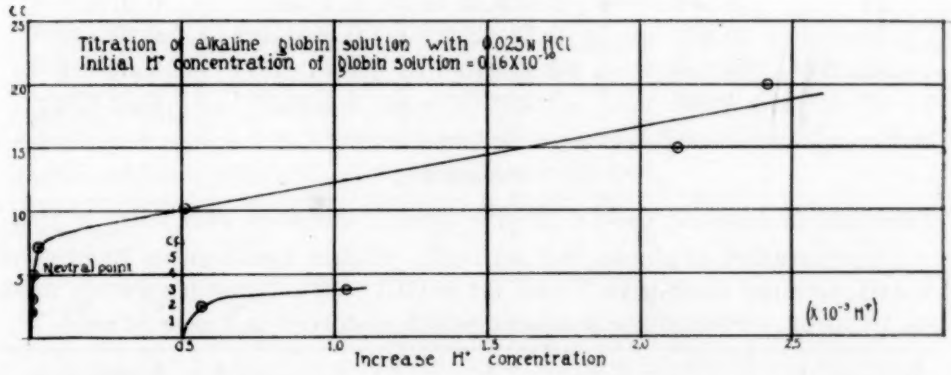
Titration of alkaline globin solution with 0.025 N HCl
Initial H' concentration of globin solution = 0.16X10⁻¹⁰
Neutral point
(X 10⁻³ H')
Increase H' concentration

FIG. 4.

the titration the protein was precipitated and redissolved by excess of acid or alkali respectively. The results are given in

[35] Hardy, W. B., *J. Physiol.*, 1905–06, xxxiii, 251.

[36] Pauli, W., *Beitr. chem. Phys. u. Path.*, 1906, vii, 531.

[37] Hofmeister, F., *Arch. exp. Path.*, 1888, xxiv, 247; 1889, xxv, 1; 1890, xxvii, 395; 1891, xxviii, 210.

[38] Galeotti, G., *Z. physiol. Chem.*, 1903–04, xl, 492; 1904, xlii, 330.

[39] Robertson, Die physikalische Chemie der Proteine, Dresden, 1912, 84.

Tables IV and V and the changes in H^+ and OH^- concentrations graphically represented in Figs. 3 and 4. Since these reactions involve direct changes in H^+ and OH^- concentrations and

TABLE IV.

Titration of Acid Globin (Preparation II) Solution with 0.025 N NaOH.

Concentration of globin, 0.2 per cent. Globin dissolved in water without addition of acid or alkali. 50 cc. globin solution titrated. Precipitate produced which dissolved in excess of alkali.

NaOH solution added.	E.M.F.	H^+	OH^-	Possible error in H^+
cc.	*volts*			
0	0.499	0.15×10^{-2}	4.3×10^{-12}	$\pm0.01\times10^{-2}$
2	0.534	0.37×10^{-3}	1.7×10^{-11}	0.02×10^{-3}
3	0.578	0.64×10^{-4}	1.0×10^{-10}	0.04×10^{-4}
4	0.632	0.75×10^{-5}	0.86×10^{-9}	0.03×10^{-5}
5	0.662	0.23×10^{-5}	2.8×10^{-9}	0.01×10^{-5}
6	0.757	0.51×10^{-7}	1.3×10^{-7}	0.02×10^{-7}
7	0.840	0.19×10^{-8}	3.5×10^{-6}	0.01×10^{-8}
8	0.893	0.22×10^{-9}	2.9×10^{-5}	0.01×10^{-9}
10	0.941	0.33×10^{-10}	2.0×10^{-4}	0.01×10^{-10}
12	0.961	0.15×10^{-10}	4.3×10^{-4}	0.01×10^{-10}
15	0.979	0.72×10^{-11}	0.89×10^{-3}	0.03×10^{-11}
19	0.989	0.48×10^{-11}	1.3×10^{-3}	0.02×10^{-11}
27	1.005	0.26×10^{-11}	2.5×10^{-3}	0.01×10^{-11}

TABLE V.

Titration of Alkaline Globin (Preparation I) Solution with 0.025 N HCl.

Concentration of globin, 0.2 per cent. Globin dissolved in 2 cc. 0.1 N NaOH, titrated back with 1.5 cc. 0.1 N HCl. 50 cc. globin solution used for titration. Precipitate produced which dissolved in excess of acid.

HCl solution added.	E.M.F.	H^+	OH^-	Possible error in H^+
cc.	*volts*			
0	0.959	0.16×10^{-10}	4.0×10^{-4}	$\pm0.01\times10^{-10}$
2	0.920	0.76×10^{-10}	0.84×10^{-4}	0.03×10^{-10}
3	0.870	0.56×10^{-9}	1.2×10^{-5}	0.02×10^{-9}
5	0.747	0.76×10^{-7}	0.85×10^{-7}	0.03×10^{-7}
7	0.609	0.19×10^{-4}	3.4×10^{-10}	0.02×10^{-4}
10	0.525	0.53×10^{-3}	1.2×10^{-11}	0.02×10^{-3}
15	0.490	0.22×10^{-2}	3.0×10^{-12}	0.01×10^{-2}
20	0.487	0.24×10^{-2}	2.6×10^{-12}	0.01×10^{-2}

true compounds between protein and acid and alkali are formed, we obtain curves differing from those in which salting out of protein has taken place. The titration curves of acid and alkaline globin are very similar to the titration curve of hydrochloric acid by sodium hydroxide given by Böttger[40] and cited by Hildebrand,[41] except that a different neutral point is obtained. This is to be expected, since globin can act either as an acid or a base. Thus in titrating acid globin with alkali, on approaching the neutral point, globin will become acid and will neutralize alkali. The sudden shift in the slope of the curve near the neutral point indicates compound formation and serves as a criterion for the determination of compound formation, whether the compound is precipitated or remains in solution. This will be shown even better in other curves. That the first part of the curve is not a straight line but appears to be so, since the changes in H^+ or OH^- concentration are small as compared with the rest of the curve, is shown on magnifying the first portion of the titration curve.

TABLE VI.

Titration of Alkaline Globin (Preparation I) Solution with a Solution of Casein (Neutral to Phenolphthalein).

Concentration of globin, 0.5 per cent. 0.25 gm. globin dissolved by 8 cc. 0.1 N KOH, then titrated back with 6.9 cc. 0.1 N HCl. Concentration of casein in titrating solution, 0.5 per cent. 0.25 gm. casein dissolved by 4 cc. 0.1 N KOH, then titrated back with 2 cc. 0.1 N HCl. 50 cc. globin solution titrated. No precipitate produced.

Casein solution added.	E.M.F.	H^+	OH^-	Possible error in H^+
cc.	*volts*			
0	0.894	0.21×10^{-9}	3.0×10^{-5}	$\pm0.02\times10^{-9}$
0.5	0.892	0.23×10^{-9}	2.8×10^{-5}	0.02×10^{-9}
1.5	0.888	0.27×10^{-9}	2.4×10^{-5}	0.02×10^{-9}
2.5	0.883	0.33×10^{-9}	1.9×10^{-5}	0.02×10^{-9}
3.5	0.880	0.38×10^{-9}	1.7×10^{-5}	0.03×10^{-9}
8.5	0.869	0.58×10^{-9}	1.1×10^{-5}	0.04×10^{-9}
13.5	0.861	0.80×10^{-9}	0.80×10^{-5}	0.06×10^{-9}
18.5	0.848	0.13×10^{-8}	4.8×10^{-6}	0.01×10^{-8}
23.5	0.836	0.22×10^{-8}	3.0×10^{-6}	0.02×10^{-8}
28.5	0.825	0.34×10^{-8}	1.9×10^{-6}	0.03×10^{-8}

[40] Böttger, W., *Z. physik. Chem.*, 1897, xxiv, 281.

[41] Hildebrand, J. H., *J. Am. Chem. Soc.*, 1913, xxxv, 854.

TABLE VII.

Titration of Alkaline Globin (Preparation I) Solution with a Solution of Casein (Neutral to Litmus).

Concentration of globin, 0.5 per cent. 0.25 gm. globin dissolved by 8 cc. 0.1 N KOH, then titrated back with 7.1 cc. 0.1 N HCl. 50 cc. globin solution titrated. Concentration of casein in titrating solution, 0.5 per cent. 0.25 gm. casein dissolved by 3 cc. 0.1 N KOH, then titrated back with 1.5 cc. 0.1 N HCl. Slight precipitate produced.

Casein solution added.	E.M.F.	H^+	OH^-	Possible error in H^+
cc.	*volts*			
0	0.843	0.17×10^{-8}	3.9×10^{-6}	$\pm0.02\times10^{-8}$
1	0.833	0.24×10^{-8}	2.6×10^{-6}	0.01×10^{-8}
2	0.831	0.26×10^{-8}	2.4×10^{-6}	0.02×10^{-8}
3	0.821	0.40×10^{-8}	1.6×10^{-6}	0.03×10^{-8}
4	0.820	0.41×10^{-8}	1.6×10^{-6}	0.03×10^{-8}
5	0.818	0.45×10^{-8}	1.4×10^{-6}	0.04×10^{-8}
10	0.804	0.78×10^{-8}	0.82×10^{-6}	0.06×10^{-8}
15	0.803	0.81×10^{-8}	0.79×10^{-6}	0.06×10^{-8}
25	0.801	0.88×10^{-8}	0.73×10^{-6}	0.07×10^{-8}

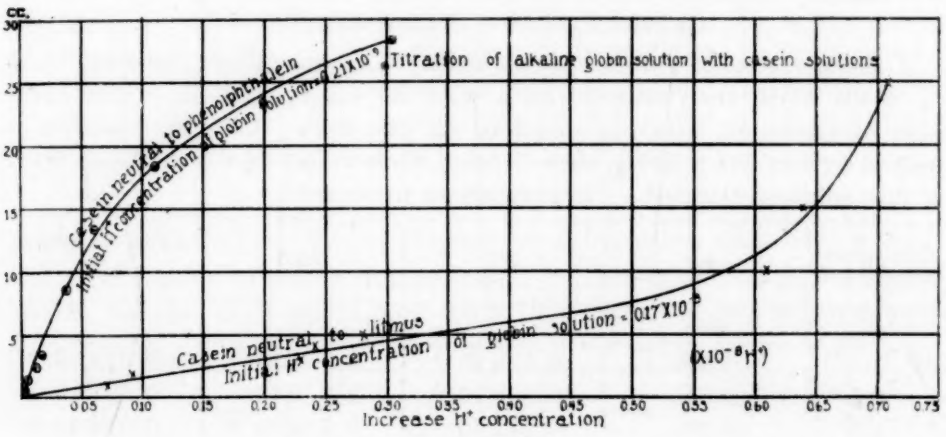
cc.
Titration of alkaline globin solution with casein solutions
Casein neutral to phenolphthalein
Initial H⁺ concentration of globin solution = 21X10⁻⁸
Casein neutral to litmus
Initial H⁺ concentration of globin solution = 017X10⁻⁸
(X10⁻⁸ H⁺)
Increase H⁺ concentration

FIG. 5.

As previously stated, both Bang and Robertson obtained compounds of globin and casein which they state were precipitated in alkaline solution. On attempting to duplicate this work, no precipitate was obtained though a compound between globin and casein is formed as shown by the data in Tables VI and VII and represented graphically in Fig. 5. The compound formed is apparently soluble. However, on titrating an acid solution of

globin with a solution of casein (neutral to phenolphthalein) a precipitate was obtained and the titrating curve (see data in Table VIII and Fig. 6) shows that a compound was formed.

TABLE VIII.

Titration of Acid Globin (Preparation II) Solution with a Solution of Casein.

Concentration of globin, 0.2 per cent. Globin dissolved in water and 1 cc. 0.1 N NaOH added. Concentration of casein (neutral to phenolphthalein), 0.5 per cent. 50 cc. globin solution titrated. Precipitate produced.

Casein solution added.	E.M.F.	H^+	OH^-	Possible error in H^+
cc.	*volts*			
0	0.552	0.18×10^{-3}	3.5×10^{-11}	$\pm0.01\times10^{-3}$
1	0.577	0.67×10^{-4}	0.96×10^{-10}	0.03×10^{-4}
2	0.609	0.19×10^{-4}	3.4×10^{-10}	0.01×10^{-4}
4	0.614	0.15×10^{-4}	4.2×10^{-10}	0.01×10^{-4}
6	0.623	0.11×10^{-4}	6.0×10^{-10}	0.01×10^{-4}
8	0.637	0.61×10^{-5}	1.1×10^{-9}	0.03×10^{-5}
11	0.650	0.36×10^{-5}	1.8×10^{-9}	0.02×10^{-5}
16	0.685	0.90×10^{-6}	0.71×10^{-8}	0.04×10^{-6}
21	0.707	0.37×10^{-6}	1.7×10^{-8}	0.03×10^{-6}

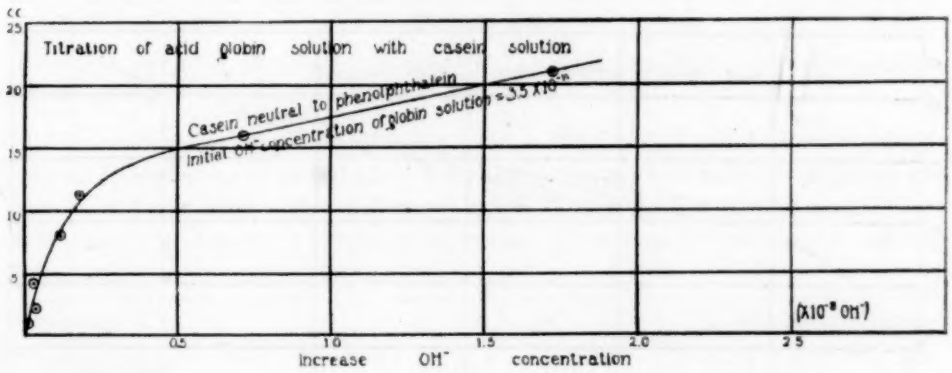

FIG. 6.

Apparently Robertson had used Globin Preparation II, which, being precipitated from an acid solution by alcohol and ether, was the acid compound of globin; and by dissolving in weak alkali, Robertson had merely partly neutralized the acid combined with the globin (not enough to cause precipitation of the globin) so that his solution was still acid, instead of alkaline as one is led to believe from his description. This was likewise true of the work

of Bang, who prepared globin according to the method of Schulz.[42] Determination of the initial acidity of the globin solution would have served as a criterion for the duplication of the work.

TABLE IX.

Titration of Acid Globin (Preparation II) Solution with a Solution of Sodium Nucleate.

Concentration of globin, 0.2 per cent. Globin dissolved in water without addition of acid or alkali. Concentration of nucleic acid, 0.4 per cent. Nucleic acid neutralized by NaOH. 50 cc. globin solution titrated. Precipitate produced.

Sodium nucleate solution added.	E.M.F.	H^+	OH^-	Possible error in H^+
cc.	*volts*			
0	0.488	0.23×10^{-2}	2.8×10^{-12}	$\pm0.01\times10^{-2}$
2	0.486	0.25×10^{-2}	2.5×10^{-12}	0.01×10^{-2}
4	0.504	0.12×10^{-2}	5.2×10^{-12}	0.01×10^{-2}
8	0.525	0.53×10^{-3}	1.2×10^{-11}	0.02×10^{-3}
12	0.536	0.34×10^{-3}	1.9×10^{-11}	0.02×10^{-3}
16	0.560	0.13×10^{-3}	4.9×10^{-11}	0.01×10^{-3}
20	0.572	0.82×10^{-4}	0.78×10^{-10}	0.03×10^{-4}
25	0.585	0.49×10^{-4}	1.3×10^{-10}	0.03×10^{-4}

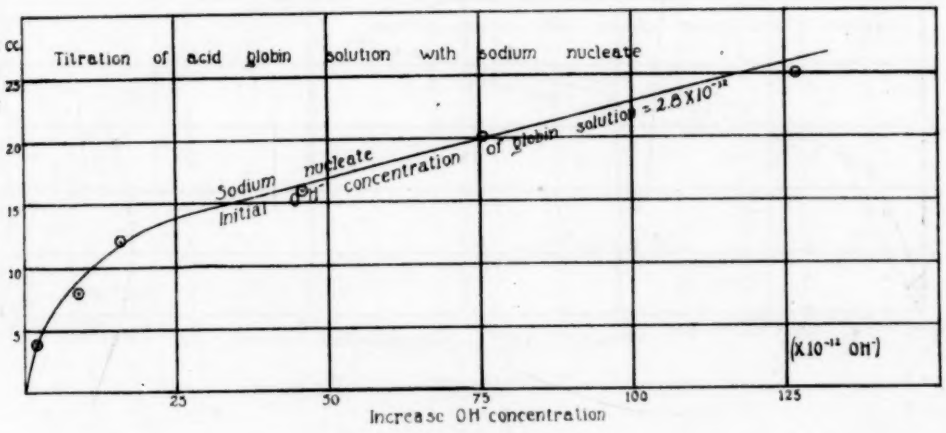

FIG. 7.

Globin also forms compounds with nucleic acid and taurocholic acid, precipitation taking place in an acid solution. The titration curve of globin with nucleic acid (see Table IX and Fig. 7) is very similar to that obtained on titration of acid glo-

[42] Schulz, F. N., *Z. physiol. Chem.*, 1898, xxiv, 449.

bin with casein. The titration curve of acid globin with bile salts (see Tables X and XI, and Fig. 8) shows a sharp break at the

TABLE X.

Titration of Acid Globin (Preparation II) Solution with a Solution of Bile Salts.

Concentration of globin, 0.2 per cent. Globin dissolved in water without addition of acid or alkali. Concentration of bile salts in aqueous solution, 0.4 per cent. Precipitate produced.

Bile salts solution added.	E.M.F.	H^+	OH^-	Possible error in H^+
cc.	*volts*			
0	0.484	0.27×10^{-2}	2.3×10^{-12}	$\pm0.01\times10^{-2}$
2	0.498	0.16×10^{-2}	4.1×10^{-12}	0.01×10^{-2}
4	0.503	0.13×10^{-2}	5.0×10^{-12}	0.01×10^{-2}
5	0.507	0.11×10^{-2}	5.9×10^{-12}	0.01×10^{-2}
7	0.511	0.93×10^{-3}	0.69×10^{-11}	0.04×10^{-3}
10	0.508	0.11×10^{-2}	6.1×10^{-12}	0.01×10^{-2}
13	0.512	0.89×10^{-3}	0.72×10^{-11}	0.04×10^{-3}
16	0.517	0.73×10^{-3}	0.88×10^{-11}	0.03×10^{-3}
24	0.540	0.29×10^{-3}	2.2×10^{-11}	0.01×10^{-3}

TABLE XI.

Titration of Acid Globin (Preparation II) Solution with a Solution of Bile Salts.

Concentration of globin, 0.2 per cent. Globin dissolved in water without addition of acid or alkali. Concentration of bile salts in aqueous solution, 0.4 per cent. Several drops 0.1 N NaOH added to the solution of bile salts to make it slightly alkaline. Precipitate produced during titration.

Bile salts solution added.	E.M.F.	H^+	OH^-	Possible error in H^+
cc.	*volts*			
0	0.515	0.79×10^{-3}	0.81×10^{-11}	$\pm0.04\times10^{-3}$
2	0.521	0.63×10^{-3}	1.0×10^{-11}	0.03×10^{-3}
5	0.525	0.53×10^{-3}	1.2×10^{-11}	0.02×10^{-3}
8	0.524	0.55×10^{-3}	1.2×10^{-11}	0.02×10^{-3}
10	0.526	0.51×10^{-3}	1.3×10^{-11}	0.02×10^{-3}
13	0.532	0.40×10^{-3}	1.6×10^{-11}	0.02×10^{-3}
17	0.536	0.34×10^{-3}	1.9×10^{-11}	0.02×10^{-3}
21	0.542	0.27×10^{-3}	2.4×10^{-11}	0.01×10^{-3}
25	0.548	0.21×10^{-3}	3.0×10^{-11}	0.01×10^{-3}

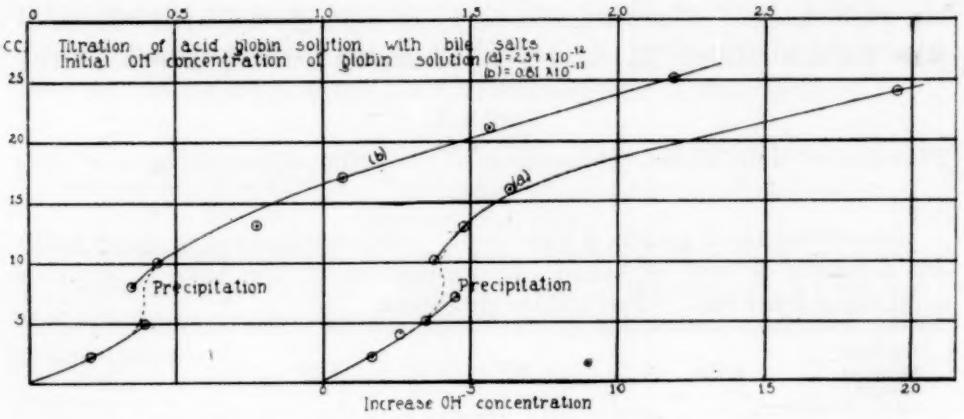
Titration of acid globin solution with bile salts
Initial OH concentration of globin solution (a) = 2.57 x 10⁻¹²
(b) = 0.61 x 10⁻¹²
cc
Precipitation
Precipitation
Increase OH concentration

FIG. 8.

point where the compound protein starts to precipitate, and the slope of the curve changes. This is to be expected since we are titrating with a mixture of two salts, sodium taurocholate and sodium glycocollate. Globin also forms a compound with deuteroalbumose, the latter acting as a weak acid. The titration curve (Fig. 9 and Table XII) indicates the formation of a compound, the proportion being roughly two parts of globin to one of albumose.

TABLE XII.

Titration of Alkaline Globin (Preparation I) Solution with a Solution of Deuteroalbumose.

Concentration of globin, 0.5 per cent. 0.25 gm. globin dissolved by 8 cc. 0.1 N KOH, then titrated back with 7.1 cc. 0.1 N HCl. Concentration of deuteroalbumose, 0.6 per cent. 50 cc. globin solution titrated. Precipitate produced.

Albumose solution added.	E.M.F.	H^+	OH^-	Possible error in H^+
cc.	*volts*			
0	0.835	0.23×10^{-8}	2.8×10^{-6}	$\pm0.02\times10^{-8}$
1	0.813	0.54×10^{-8}	1.2×10^{-6}	0.04×10^{-8}
2	0.792	0.13×10^{-7}	5.1×10^{-7}	0.01×10^{-7}
4	0.793	0.12×10^{-7}	5.3×10^{-7}	0.01×10^{-7}
7	0.792	0.13×10^{-7}	5.1×10^{-7}	0.01×10^{-7}
15	0.786	0.16×10^{-7}	4.0×10^{-7}	0.01×10^{-7}
20	0.779	0.21×10^{-7}	3.0×10^{-7}	0.01×10^{-7}

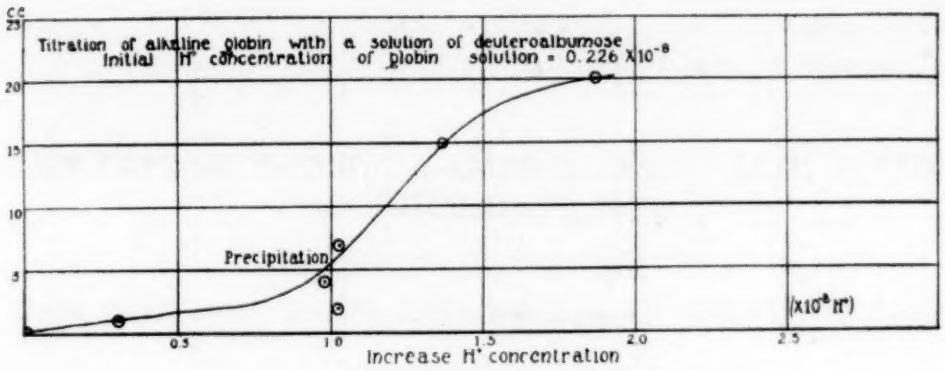

FIG. 9.

SUMMARY.

1. Use of the gas chain has been made to follow the changes in the H^+ and OH^- concentration in titrating acid and alkaline solutions of globin with solutions of other proteins and of precipitating inorganic salts.

2. Titration curves showing the changes in H^+ and OH^- concentration during titration have been plotted which indicate that true protein compounds of globin are formed, some of which may precipitate at a proper acidity or alkalinity, while others may be soluble.

3. A distinction can be made by means of the gas chain between the salting out of globin from solution by inorganic salts and the precipitation of a compound protein.

4. Determination of the H^+ or OH^- concentration in protein solutions used in the preparation of compound proteins serves as a criterion for the duplication of such work.

I am indebted to Professors F. P. Gay and T. Brailsford Robertson for the interest taken in this work, and to the George Williams Hooper Foundation for Medical Research for financial aid in carrying out the investigation.

THE FATE OF NORMAL α-AMINOCAPROIC ACID IN THE PHLORHIZINIZED DOG.

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(From the Harriman Research Laboratory, Roosevelt Hospital, New York.)

(Received for publication, March 24, 1916.)

As a result of the work of Lusk,[1] Ringer,[1] and Dakin,[2] we know that a number of the amino-acids obtained from proteins can give rise to an increased excretion of glucose when administered to phlorhizinized dogs. One of the amino-acids that has not yet been investigated is normal α-aminocaproic acid, or norleucine, first isolated from brain tissue by Abderhalden and Weil.[3]

It is the purpose of this paper to present the results of an attempt to ascertain the effect of the administration of this substance upon the excretion of nitrogen and glucose in the phlorhizinized dog. Two experiments were made with each of the dextrorotatory and racemic acids and one with the levorotatory acid.

EXPERIMENTAL.

Phlorhizin was administered in daily doses of 1 gm. each in 7 or 8 cc. of cottonseed oil. In Experiment 5 the dog was prepared with epinephrin, as recommended by Sansum and Woodyatt.[4] The urine was collected by catheter and the bladder washed with sterile 2 per cent boric acid solution at 12 hour intervals. Glucose was determined according to Benedict,[5] by polarization before and after fermentation and by fermentation (Lohnstein), gross urea nitrogen by the urease method,[6] and

[1] Literature reviewed by Lusk, G., *Ergebn. Physiol.*, 1912, xii, 315.

[2] Dakin, H. D., *J. Biol. Chem.*, 1913, xiv, 321.

[3] Abderhalden, E., and Weil, A., *Z. physiol. Chem.*, 1913, lxxxiv, 39.

[4] Sansum, W. D., and Woodyatt, R. T., *J. Biol. Chem.*, 1915, xxi, 1.

[5] Benedict, S. R., *J. Biol. Chem.*, 1911, ix, 57.

[6] It should be noted that it was necessary to allow a much longer time (3 hours) for the action of the urease in these urines than is recommended by Plimmer and Skelton (*Biochem. J.*, 1914, viii, 70).

Period.	Substance administered.	Nitrogen.	Glucose.			G:N	Extra glucose.	Urea and NH_3 N.		Amino nitrogen.		Acetone.	β-Hydroxybutyric acid.	Remarks.
			Benedict.	Polarization.	Fermentation.				Per cent of total N.		Per cent of total N.			
		gm.	gm.	gm.	gm.		gm.	gm.		gm.		gm.	gm.	
Experiment 1.														
2		2.207	6.48	6.6	6.6	2.94		1.988	90.1			0.179	1.277	8 hrs.
3		3.552	10.50	10.3	10.8	2.95		3.137	88.3	0.116	3.27	0.288	2.138	
4	**3.60** gm.	**3.937**	**13.60**	**14.2**	**14.0**	**3.45**	**3.94**	**3.320**	**84.3**	**0.186**	**4.72**	**0.455**	**2.095**	Weight 5 kg.
5	dl-acid.	**2.930**	**10.30**	**11.0**		**3.51**		**2.630**	**89.8**	**0.092**	**3.14**	**0.399**	**1.395**	11 hrs.
6		6.153	20.60	20.6		3.35		5.543	90.1			0.760	2.933	24 "
Experiment 2.														
4		3.045	10.50	9.05	10.6	3.45		2.764	90.8	0.051	1.67	0.143	0.822	
5		3.243	10.90	9.34	10.9	3.36		3.064	94.5			0.180	0.852	
6	**5.4** gm. d-	**3.504**	**14.01**	**12.6**	**13.8**	**4.00**	**3.89**	**3.255**	**92.9**	**0.042**	**1.19**	**0.190**	**0.672**	Weight 5 kg.
7	acid.	**3.711**	**13.17**	**11.8**	**13.5**	**3.55**		**3.474**	**93.6**	**0.047**	**1.28**	**0.117**	**0.444**	
8		3.262	11.27	10.2	11.4	3.46		3.137	96.2	0.024	0.71	0.069		
9		2.680	8.60			3.21		2.540	94.8	0.042	1.56	0.065		
Experiment 3.														
3		4.225	13.94		14.2	3.30		3.931	93.1	0.047	1.10	0.298	1.330	
4		5.031	15.94	16.5	15.6	3.16		4.566	90.8	0.116	2.30	0.293	1.250	
5		4.464	14.90	14.1	14.8	3.34		4.092	91.7	0.074	1.65	0.416	1.684	
6	**8.25** gm. l-	**4.807**	**18.50**	**18.3**	**19.1**	**3.85**	**8.51**	**4.603**	**95.7**	**0.105**	**2.18**	**0.612**	**2.395**	Weight 9.1 kg.
7	acid.	**4.350**	**17.53**	**18.1**	**18.0**	**4.03**		**4.194**	**96.4**	**0.079**	**1.86**	**0.579**	**1.880**	
8		5.098	17.85	18.0	17.5	3.50		4.752	93.2	0.080	1.58	0.517	1.162	
9		4.495	14.95		14.7	3.35		4.121	91.4					

8		5.098	17.85	18.0	17.5	3.50		4.752	93.2	0.080	1.58	0.517	1.162	
9		4.495	14.95		14.7	3.35		4.121	91.4					
Experiment 4.														
1		2.555	7.07	6.58	6.99	2.77		2.284	89.4	0.025	0.83			
2		3.506	9.58	9.35	9.31	2.73		3.096	88.3	0.024	0.69			
3	5.76 gm. d-acid.	2.992	9.72	9.55	9.72	3.25	3.45	2.510	83.9	0.090	3.1			Weight 4.6 kg.
4		2.710	9.41	9.70	9.52	3.49		2.346	87.3	0.049	1.8			
Experiment 5.														
4		4.580	15.67	15.6	15.5	3.42		4.174	91.1	0.048	1.04			6 hrs. 3 mg. epinephrin twice.
5		5.291	19.06	19.8	19.8	3.61		4.898	92.6			0.076	0.616	7 hr. 3 mg. epinephrin.
6		7.614	24.76	24.3	24.6	3.25		6.953	91.2	0.210	3.42	0.099	0.617	11 hrs.
7	14.7 gm. dl-acid.	9.350	28.48	27.0	30.4	3.05	3.6	8.253	88.3	0.655	7.00	0.142	0.949	12 hrs. Weight 11.2 kg.
8		9.970	32.73	31.1	34.6	3.28		8.929	89.5	0.305	3.06	0.108	0.497	
9		10.08	32.47	30.4	33.0	3.22		9.402	93.4	0.255	2.53	0.091	0.441	
10		8.954	29.11	28.1	29.7	3.25		7.984	89.2	0.245	2.74	0.091	0.573	

aminonitrogen by the method of Benedict and Murlin.[7] In all but Experiment 5 the acetone was determined by titration with iodine after distillation and the β-hydroxybutyric acid by Shaffer's method applied to the ethereal extract. In Experiment 5 Shaffer's method[8] was used directly.

Preparation of the Normal α-Aminocaproic Acid.—The amino-acid was prepared by the method described by Abderhalden, Froehlich, and Fuchs.[9] There were obtained 290 gm. of bromocaproic acid from 192 gm. of caproic acid but only 60 gm. of recrystallized normal α-aminocaproic acid. This is a much smaller yield than that reported by Abderhalden, Froehlich, and Fuchs. The melting point given by them is 297–300°. This preparation melted at 294–296° and contained 10.55 per cent nitrogen (calculated, 10.66 per cent). The formyl derivative was then prepared and the optical isomers separated by means of their brucine salts. The free amino-acids were isolated in the usual manner.

Normal d-α-Aminocaproic Acid.—0.5427 gm. in 12.5 cc. HCl (s.g. 1.11), in a 2 dm. tube, rotated the plane of polarization + 1.48°, $[\alpha]_D$ + 17.05°. The substance was, therefore, made up of 84.1 per cent pure *d*-acid and 15.9 per cent *l*-acid.[10]

Normal l-α-Aminocaproic Acid.—0.4823 gm. in 12.5 cc. HCl (s.g. 1.11), in a 2 dm. tube, rotated the plane of polarization −1.74°, $[\alpha]_D$ − 22.6°. This preparation was, therefore, 91.6 per cent pure *l*-acid and 8.4 per cent *d*-acid.[10]

The amino-acid was administered subcutaneously in three or four doses at intervals of 1 or 2 hours. Attempts were made to isolate unchanged amino-acid from the urine by means of its insoluble copper salt. In only one experiment was this successful. In calculating the amount of glucose derived from the administered substance, it was assumed, as it has been by previous workers, that all of the nitrogen of the amino-acid appeared in the urine in the experimental period, or in that immediately following.

[7] Benedict, S. R., and Murlin, J. R., *J. Biol. Chem.*, 1913–14, xvi, 386.

[8] Shaffer, P. A., and Marriott, W. McK., *J. Biol. Chem.*, 1913–14, xvi, 276.

[9] Abderhalden, E., Froehlich, C., and Fuchs, D., *Z. physiol. Chem.*, 1913, lxxxvi, 454.

[10] Schulze, E., and Likiernik, A., *Z. physiol. Chem.*, 1893, xvii, 523.

DISCUSSION.

The results of the experiments indicate that both optical isomers are attacked in the organism of the dog, the dextro form being only slightly more resistant. The amount of "extra glucose" excreted was, in four experiments, approximately equal to that of the amino-acid administered. In the two instances in which the amount of extra glucose was smaller than this, there was unquestionably a considerable excretion of unchanged amino-acid. In Experiment 4, in which 3.45 gm. of extra glucose were obtained from 5.76 gm. of the *d*-acid, the increase in the amino nitrogen of the urine indicated that 15 per cent of the amino-acid appeared in the urine unchanged. In the one experiment in which the dog was prepared with epinephrin, only 3.6 gm. of extra glucose were obtained after the administration of 14.7 gm. of *dl*-acid. Unfortunately the amount of amino-acid available does not permit of a repetition of the experiment. However, it is scarcely possible that the extra glucose obtained in the other experiments should have been derived from the glycogen remaining in the body after 3 or 4 days' treatment with phlorhizin. Normal α-aminocaproic acid has no narcotic or other toxic action. It seems quite as likely that treatment with epinephrin interferes with the formation of glucose from some amino-acids. The low ratios reported by Sansum and Woodyatt[4] indicate that this may be the case. Certainly a considerable quantity of unchanged amino-acid was eliminated in the urine in this experiment. 35 cc. of the urine of the experimental period (total volume 800 cc.) were precipitated with $CuSO_4$, keeping the reaction neutral. The precipitate was filtered out, washed, and decomposed with H_2S. The liquid was filtered from the CuS, evaporated, and treated with five volumes of alcohol. Characteristic crystals of the amino-acid were obtained. These were filtered out, washed, dissolved in water, and the copper salt was again precipitated. Its appearance was quite characteristic. It weighed 88 mg. Unfortunately the material was lost in the process of analysis but there can be little doubt that it was the copper salt of normal α-aminocaproic acid. The amount recovered corresponds, in the urine of the entire period, to 1.3 gm. of amino-acid, or 9 per cent of the amount injected. It is almost certain that a larger amount was excreted

but failed of recovery. The increase in amino nitrogen in the urine of the experimental and following periods was approximately 0.4 gm., indicating the presence of at least 25 per cent of the injected amino-acid. The diminished urea excretion would account for an even greater elimination of unchanged amino-acid.

Attention should also be called to the fact that in this experiment and in one other (3) there was an increased excretion of acetone and β-hydroxybutyric acid in the urine in the experimental period. It is possible that there are two paths of catabolism for *n*-aminocaproic acid, one through β-hydroxybutyric acid and the other through a possible precursor of glucose.

Another possible explanation of the result in Experiment 5, though it does not apply to the other, is that the β-hydroxybutyric acid was determined by the Shaffer method. Although experiment showed that normal α-aminocaproic acid yielded only negligible amounts of iodine-binding substances when treated by this method, it is possible that intermediate products of catabolism were present and reacted as does β-hydroxybutyric acid. In the other experiments, this possibility was largely obviated by making the determinations upon the ether extract of the urine.

No attempt has been made to picture the intermediate stages in the formation of glucose from aminocaproic acid. However, it would seem that at least four of the six carbon atoms of the amino-acid appear as glucose.

THE ADMISSIBILITY OF AMMONIUM MAGNESIUM PHOSPHATE AS A FORM IN WHICH TO WEIGH PHOSPHORIC ACID.

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A procedure has been employed in this laboratory by which the numerical index of nucleotides may be indirectly determined provided that the nucleotide contains both purine and pyrimidine groups. The essential feature of this procedure consists in determining the "phosphorus partition" of the nucleotide, which can be done with the greatest accuracy, and which gives values that lead directly to the index sought.

The method was first studied in its application to various preparations of yeast nucleic acid,[1] and, as may have been expected, the substance was found to be a tetra-nucleotide. An extension of the method to other nucleotides has in every case led sharply to the index of the nucleotide examined, and in some instances very surprising results have been obtained. All these results are based upon determinations of phosphoric acid in which the acid was weighed as magnesium ammonium phosphate, and it remains to show that values for phosphoric acid obtained in this way are correct.

The countless number of phosphorus determinations that are recorded in the literature and the much greater number of unreported analyses which have been made in the industries would lead one to believe that an advantageous change even in a detail of the method employed could not be suggested. The oxidation of the substance to be analyzed for the destruction of organic matter and precipitation with ammonium molybdate for the removal of bases are two preliminary steps in a phosphoric acid

[1] Jones, W., and Riley, C., *J. Biol. Chem.*, 1916, xxiv, p. iii.

estimation that are employed or not as the case requires. But in any event the phosphoric acid is finally precipitated as magnesium ammonium phosphate, which is decomposed by heating and weighed as magnesium pyrophosphate. From the weight of the pyrophosphate the corresponding weight of phosphoric acid is calculated.

The uniform rejection by analysts of such a substance as crystalline magnesium ammonium phosphate in favor of magnesium pyrophosphate, in spite of the fact that the one substance precedes the other in the analytical scheme and must of necessity transmit to the other any errors committed in its manipulation, is a matter that renders one suspicious and suggests caution. Nevertheless, the following data demonstrate very clearly that if there is any choice between the two substances, aside from economy of time, it is in favor of the crystalline compound.

To prove the admissibility of magnesium ammonium phosphate it is necessary, and sufficient, to establish three points: (a) that magnesium ammonium phosphate can be brought to a constant weight; (b) that the substance can be easily and completely removed from a filter paper when dry; (c) that the substance possesses exactly the composition expressed by the formula $MgNH_4PO_4.6H_2O$ or at least that it will produce the exact amount of magnesium pyrophosphate that this formula demands. These three points are taken up in order as follows.

a. In order to meet the conditions of a phosphorus partition study the specimens of magnesium ammonium phosphate employed in this work were prepared from the hydrolytic products of yeast nucleic acid. Five portions of the substance were heated with 5 per cent sulfuric acid from 1 to 5 hours and after removal of the guanine with ammonia, the phosphoric acid was precipitated with magnesia mixture, using such precaution as to insure the formation of crystalline magnesium ammonium phosphate. The five precipitates were filtered on ashless papers, washed carefully, allowed to dry in the room for 19 hours, and weighed. In order to see if any further change in weight would occur at the room temperature, the filters were allowed to stand several days longer and weighed from time to time. The results are given in Table I.

TABLE I.

	Weights of filter papers containing magnesium ammonium phosphate dried at room temperature.				
	I.	II.	III.	IV.	V.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
After 19 hrs.....	0.7297	0.7535	0.7882	0.7695	0.7254
" 5 " longer.....	0.7284	0.7527	0.7875	0.7690	4.6669
" 21 " "	0.7271	0.7514	0.7864	0.7679	4.6668
" 8 " "	0.7268	0.7510	0.7860	0.7677	4.6668
" 17 " "	0.7272	0.7212	0.7863	0.7680	4.6668

It will be seen that up to the last period a slow but continual loss in weight has occurred uniformly in all the specimens. But this weight is lost from the filter paper, not from the precipitate, for at the beginning of the second period, most of Precipitate V was transferred to a platinum crucible lid and weighed. The constancy of this weight throughout shows that in the other four specimens it was not the magnesium ammonium phosphate whose weight altered.

This change in the weight of a filter paper, which may amount to 2 mg. in 24 hours, can scarcely affect the results obtained by weighing an empty filter paper a few minutes after the phosphate precipitate has been dusted off. It seems to be caused by a change of atmospheric conditions (temperature and moisture).

The following series shows that the filters may increase in weight at the room temperature. No. VI is a total phosphorus determination after all organic matter had been destroyed.

TABLE II.

	I.	II.	III.	IV.	V.	VI.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
After 16 hrs. in air...	0.9516	0.8327	0.8618	0.8185	0.7400	1.0386
After 24 hrs. longer in air.....	0.9528	0.8337	0.8631	0.8201	0.7419	1.0395

The next series shows that the filters may increase in weight even when they are allowed to remain in the thermostat at 40° for 48 hours. No. VI is a determination of total phosphorus and No. V which remained nearly constant, was weighed on a platinum crucible lid.

TABLE III.

	I.	II.	III.	IV.	V.	VI.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
After 16 hrs. in air...	0.8241	0.8342	0.8000	0.7903	5.1175	1.0619
2 days longer at 40°..	0.8250	0.8352	0.8011	0.7912	5.1171	1.0630

The figures show that while a washed filter paper may vary slightly, ammonium magnesium phosphate maintains a constant weight when allowed to remain in the air or even at a temperature of 40°. The results given are extreme cases. As a rule the filter papers do not vary either way as much as 1 mg. in 24 hours.

b. In order to find whether small particles adhere to the filter when crystalline magnesium ammonium phosphate is removed, ten of the filters were dusted free from the salt and ashed in a weighed porcelain crucible. The total ash weighed 17 mg. or about 1.7 mg. for each filter paper. The ash was dissolved by long digestion with hot nitric acid and from the diluted solution the phosphoric acid was precipitated by the addition of ammonium nitrate and treatment with ammonium molybdate. The yellow ammonium phosphomolybdate was converted into magnesium ammonium phosphate which weighed about 5 mg. or about 0.5 mg. for each filter paper. Thus, if one uses an ashless filter paper and determines the phosphoric acid as magnesium pyrophosphate the final weighing is liable to be 1.7 mg. too high even though the paper has been well washed. Less than one-third of this error will be caused by the adhesion of magnesium ammonium phosphate to a filter paper.

c. As far as the composition of magnesium ammonium phosphate is of concern in this connection, it is only necessary to show that the substance produces the amount of magnesium pyrophosphate required by the equation:

$$2NH_4MgPO_4.6H_2O = Mg_2P_2O_7 + 13H_2O + 2NH_3$$

Specimens of magnesium ammonium phosphate obtained in such experiments as those described above were heated to a constant weight in a weighed porcelain crucible. The determinations were made successively in the same crucible, and the negligible differ-

ences found are probably due to the difficulty of weighing the porcelain crucible.

TABLE IV.

	I.	II.	III.	IV.	V.
Magnesium ammonium phosphate used, gm....	0.3772	0.5619	0.4848	0.4467	0.5741
Magnesium pyrophosphate obtained, gm....	0.1703	0.2542	0.2642	0.2013	0.2594
Percentage found.....	45.15	45.24	45.18	45.06	45.20
Percentage required.....	45.09	45.09	45.09	45.09	45.09

The results given in this paper seem to show that in the quantitative determination of phosphoric acid, the conversion of magnesium ammonium phosphate into magnesium pyrophosphate is a superfluous operation.

After this paper had been sent to the publishers, a letter was received from Dr. Louis Baumann (State University of Iowa), which contained the following data.

The magnesium contained in a solution prepared by dissolving pure magnesium ribbon in dilute hydrochloric acid was precipitated as the triple phosphate, then filtered on a weighed ash-free filter paper. The precipitate and paper were dried for 48 hours in the laboratory atmosphere. Results in triplicate:

$MgNH_4PO_4.6H_2O$	
Calculated for 0.0155 magnesium.	Found.
0.1564	0.1566
	0.1565
	0.1561

HYDROLYSIS OF YEAST NUCLEIC ACID WITH AMMONIA.

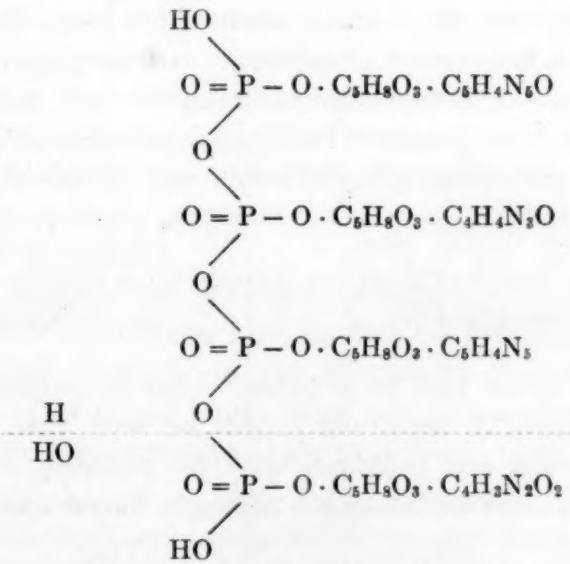
By WALTER JONES and HILDEGARDE C. GERMANN.

(*From the Laboratory of Physiological Chemistry, Johns Hopkins University, Baltimore.*)

(Received for publication, April 1, 1916.)

About two years ago, Thannhauser[1] studied the action of duodenal juice on yeast nucleic acid and found that a tri-nucleotide is formed which has the composition represented by the formula $C_{32}H_{49}P_3O_{23}N_{15}$, is levorotatory to polarized light, and forms a crystalline brucine salt of the composition $C_{32}H_{49}P_3O_{23}N_{15}(C_{23}H_{26}N_2O_4)_6$ which melts at 200–205°. From the tri-nucleotide he prepared the three nucleosides guanosine, adenosine, and cytidine.

The article is written with a little caution, evidently because the analytical values of the substance do not lead to the formula that we would assign to a tri-nucleotide derived from yeast nucleic acid. Thannhauser therefore names his substance *Triphosphonucleinsäure.* But if he holds the prevailing well grounded opinion of the constitution of yeast nucleic acid he must believe that his substance is formed from yeast nucleic acid according to the following scheme,

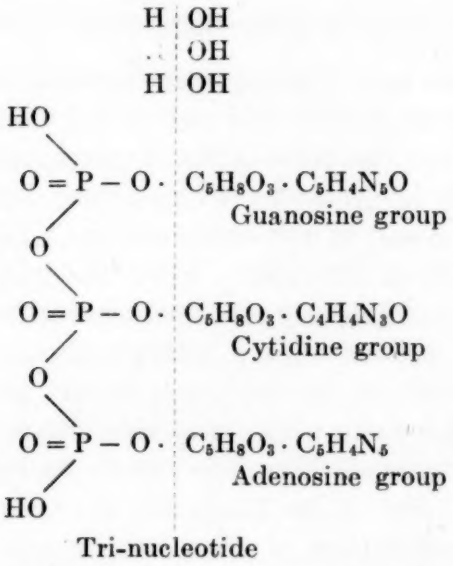


Yeast nucleic acid

[1] Thannhauser, S. J., *Z. physiol. Chem.*, 1914, xci, 329.

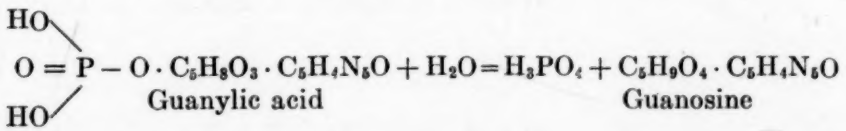
in which yeast nucleic acid is represented as decomposing by hydrolysis into a tri-nucleotide which contains the guanine, cytosine, and adenine groups, and a mono-nucleotide which contains the uracil group.

The formation of the three nucleosides from the tri-nucleotide must be represented as follows.



The following criticism of Thannhauser's results will make much clearer the matters that are to be discussed in this paper.

(a) The preparation of three nucleosides from the tri-nucleotide is of no consequence if evidence can be produced to show that this supposed tri-nucleotide is guanylic acid, a mono-nucleotide which has been prepared from yeast nucleic acid by the action of ferments,[2] and which can yield only one nucleoside,



Thannhauser states that he obtained three nucleosides, but if his results are carefully examined it will be seen that he obtained neither adenosine nor cytidine, but two picrates, one of which could be picric acid as far as his nitrogen determination is concerned.

[2] Jones, W., and Richards, A. E., *J. Biol. Chem.*, 1914, xvii, 71.

(b) A tri-nucleotide containing groups of guanine ($C_5H_5N_5O$), cytosine ($C_4H_5N_3O$), and adenine ($C_5H_5N_5$) must contain thirteen atoms of nitrogen and therefore cannot be correctly represented by the formula $C_{32}H_{49}P_3O_{23}N_{15}$ which contains fifteen atoms of nitrogen.

(c) The analytical data given for the crystalline brucine salt of the tri-nucleotide answer as well for the crystalline brucine salt[3] of guanylic acid.

	Required for $C_{10}H_{14}N_5PO_8 \cdot (C_{23}H_{26}N_2O_4)_2$ (brucine salt of guanylic acid):	Required for $C_{32}H_{49}N_{15}P_3O_{23} \cdot (C_{23}H_{26}N_2O_4)_6$ (brucine salt of tri-nucleotide):	Found:
N	10.94	10.90	10.36
P	2.69	2.88	2.71
C	58.39	58.82	59.12
H	5.74	5.77	5.88

(d) The tri-nucleotide is levorotatory and forms a crystalline brucine salt which melts at 200–205°.

Guanylic acid also is levorotatory and forms a crystalline brucine salt that melts at 200–205°.

In a more recent communication, Thannhauser and Dorfmüller[4] state that the same tri-nucleotide can be prepared from yeast nucleic acid by chemical means. They heated yeast nucleic acid with ammonia in the autoclave at 120–125° and from the product they prepared a crystalline brucine salt that melts at 205° and has the composition represented by the formula $C_{32}H_{49}N_{15}P_3O_{23}(C_{23}H_{26}N_2O_4)_6$. This brucine salt is stated to be identical with the brucine salt of the tri-nucleotide that Thannhauser had previously prepared from yeast nucleic acid by the action of duodenal juice.

A second brucine salt was also obtained. Its melting point and elementary analysis are given but no formula is assigned. No examination is made of the hydrolytic products of either substance, the principal purpose of the article being to show that the same tri-nucleotide is formed from yeast nucleic acid

[3] Levene, P. A., and Jacobs, W. A., *J. Biol. Chem.*, 1912, xii, 421.

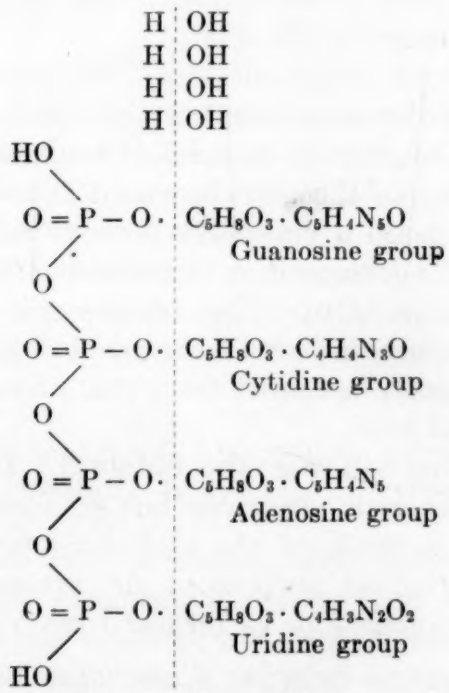
[4] Thannhauser, S. J., and Dorfmüller, G., *Z. physiol. Chem.*, 1915, xcv, 259. This number was issued Nov. 6, 1915, but has reached us only very recently.

by hydrolysis, whether the hydrolysis be brought about by ammonia in the autoclave at 120–125° or by the action of the ferments of duodenal juice.

In the last article, Thannhauser and Dorfmüller have described a method, long known in this laboratory, by which anyone can easily and quickly gain possession of the nucleotides of yeast nucleic acid. Moreover, a description has recently been given of an easily executed method by which the numerical indices of nucleotides may be found and their constitution determined.[5] We therefore wish to make a condensed statement of some of our results.

Hydrolysis of Yeast Nucleic Acid with Ammonia at High Temperatures.

Several years ago a serious attempt was made to prepare nucleosides from yeast nucleic acid by heating the substance in the autoclave with ammonia according to the method discovered and described by Levene and Jacobs.[6]



Yeast nucleic acid

[5] Jones, W., *J. Biol. Chem.*, 1916, xxiv, p. iii.

[6] Levene and Jacobs, *Ber. chem. Ges.*, 1910, xliii, 3154.

The attempt to prepare nucleosides was made to gain possession of adenosine and to use the substance in studies of the action of ferments on combined adenine.[7]

Accordingly, yeast nucleic acid was heated in the autoclave with ammonia for 3½ hours at 175–180°, and as Levene and Jacobs specially enjoin, the temperature and time of heating were rigidly observed. A black tar was formed from which, of course, adenosine could not be prepared. After a number of discouraging attempts to prepare nucleosides in this way, some pure guanosine[8] was tested to see how the substance would behave when heated in the autoclave for 3½ hours at 175–180°. The substance was completely decomposed, and it became apparent that one could not expect to obtain nucleosides by heating nucleic acid at 175–180°. The experiment with nucleic acid was therefore made at lower temperatures and the time of heating was shortened to 2 hours.

At 165° the products were badly injured.

At 160° the products were considerably injured but nucleosides could be shown present.

At 150° the results were very satisfactory and nucleosides could be obtained from the product without difficulty.

At 145° all phosphoric acid is set free and can be precipitated directly with magnesia mixture. Under these conditions the production of nucleosides must be complete and there can be no reason for heating at any higher temperature.

Below 140° the liberation of phosphoric acid is partial and is less the lower the temperature.

At 120° no phosphoric acid is liberated so that nucleotides must be exclusively formed.

At 110° no phosphoric acid is liberated and still the nucleic acid is completely decomposed into soluble products.

We have chosen 115° as the temperature favorable to the formation of nucleotides. At this temperature there is no danger of forming nucleosides.

[7] Jones, *J. Biol. Chem.*, 1911, ix, 169. Amberg, S., and Jones, W., *Z. physiol. Chem.*, 1911, lxiii, 407.

[8] Guanosine had been plentifully obtained at this time by the action of ferments on yeast nucleic acid.

Hydrolysis of Yeast Nucleic Acid with Ammonia at 115°.

A solution of 100 gm. of yeast nucleic acid in 530 cc. of 2.5 per cent ammonia was heated in an autoclave at 115° for 1½ hours. The cooled product, which was strongly alkaline and could be shown to contain no nucleic acid, was treated with 95 per cent alcohol as long as a precipitate was formed and until the clear supernatant fluid showed a tendency to become cloudy. To bring about this condition about one and three-fourths volumes of alcohol are required.

This procedure was suggested by the difference in conduct of the two di-nucleotides of yeast nucleic acid when aqueous solutions of their potassium salts are poured into a large excess of alcohol. The potassium salt of one di-nucleotide is promptly precipitated by alcohol while the potassium salt of the other di-nucleotide remains suspended as an emulsion, and by this difference in conduct of their potassium salts, the two di-nucleotides can be separated from one another.[9]

The autoclave product that we are describing consists to a considerable extent, but not entirely, of the ammonium salts of the two di-nucleotides of yeast nucleic acid, and by treatment with alcohol, in the manner described, it may be separated rather sharply into two well characterized fractions, one of which is thrown down as a heavy gray precipitate while the other remains dissolved in the alcoholic fluid, if too much alcohol has not been added. The point at which the addition of alcohol should be discontinued is quite sharply indicated by a cloudy appearance of the liquid above the precipitate. This is caused by a little of the adenine di-nucleotide which is precipitated in colloidal form, and in such a case the cloud should be redissolved by the addition of a little water.

The precipitate is referred to as the guanine-cytosine fraction and contains only substances that produce guanine by acid hydrolysis. The alcoholic fluid is referred to as the adenine-uracil fraction and contains only substances that produce adenine by acid hydrolysis.

The alcoholic solution was filtered off and the precipitate was thoroughly washed with alcohol of the same strength as the filtrate. The alcohol washings were discarded.

[9] Jones and Richards, *J. Biol. Chem.*, 1915, xx, 25.

The Guanine-Cytosine Fraction.[10]

The heavy gray precipitate, formed by the addition of alcohol to the alkaline autoclave product, was dissolved in hot water and a small amount of dark, insoluble gelatinous material was removed with the centrifuge. By applying to the clear solution the procedure with lead acetate that is described in the next section a substance was finally obtained from which could be prepared a crystalline brucine salt melting at 200–205°. This crystalline compound is the brucine salt of guanylic acid. It produces the required amount of guanine, but not a trace of adenine, by hydrolysis with sulfuric acid. Its entire phosphoric acid is set free by heating with 5 per cent sulfuric acid, which proves that the substance does not contain a pyrimidine group.

The substance is undoubtedly the brucine salt of guanylic acid. Its melting point and method of preparation show with equal clearness that it is the brucine salt of Thannhauser and Dorfmüller's *Triphosphonucleinsäure.*

The Adenine-Uracil Fraction.

The perfectly clear alcoholic solution was diluted with an equal volume of boiling water, acidified with acetic acid, and treated with 25 per cent lead acetate as long as a precipitate was formed. After the hot material had cooled, the lead compound was filtered off, suspended in hot water, and decomposed with hydrogen sulfide. It is necessary to avoid a great excess of hydrogen sulfide and to boil the product finally until the vapor does not blacken lead acetate paper. Otherwise sulfur compounds are sure to be obtained. It is in fact advisable to use a slightly insufficient amount of hydrogen sulfide gas and remove the small amount of lead from the boiling hot filtered solution by the careful addition of hydrogen sulfide water.

The final filtrate from lead sulfide was evaporated to a syrup at 50° under diminished pressure and treated with several volumes of 95 per cent alcohol. A resin was precipitated which was ground with absolute alcohol to a heavy white powder and dried in a

[10] This fraction was examined by B. E. Read. He will publish later a full account of his work.

sulfuric acid desiccator. The substance is a di-nucleotide which contains groups of adenine and uracil.

Preparations of the substance often give a trace of guanine by hydrolysis with sulfuric acid. This is because a perfect separation of the ammonium salts with alcohol as described above was not effected. In such a case the di-nucleotide is dissolved in water and after making strongly alkaline with ammonia, the solution is treated with alcohol until the clear fluid shows a tendency to become cloudy. The small precipitate, which is a part of the guanosine-cytosine fraction, is filtered off, and after acidifying with acetic acid, the solution is diluted with hot water and treated with lead acetate. From the lead compound the di-nucleotide is prepared as already outlined.

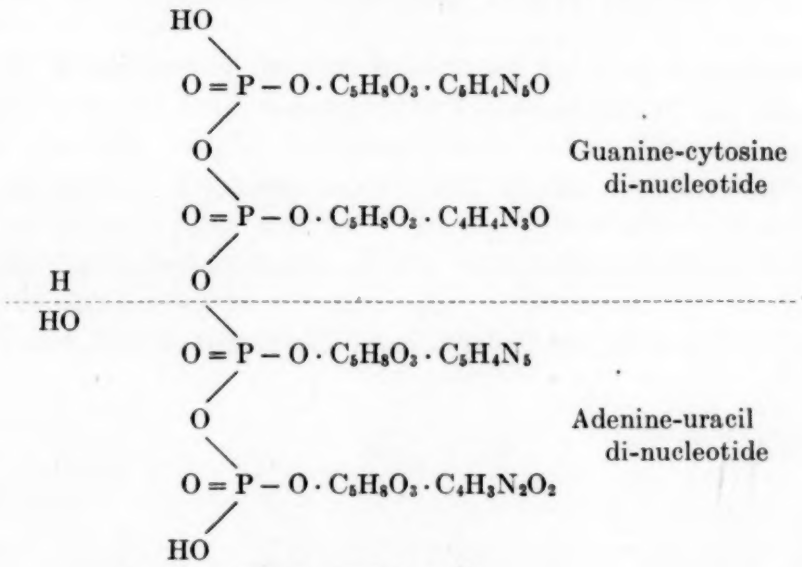
The Adenine-Uracil Di-Nucleotide.

The white granular powder prepared as described is adenine-uracil di-nucleotide and was obtained almost quantitatively from yeast nucleic acid. It contains the required amount of nitrogen and yields the required amount of adenine when hydrolyzed with dilute sulfuric acid. But it does not yield a trace of guanine. The partition of its phosphorus proves that the substance is a di-nucleotide. Exactly one-half of its phosphoric acid is easily split off and half is firmly bound; therefore as the nucleotide contains only one purine group, it can contain only one pyrimidine group.

Some interesting results were obtained with a substance that was prepared in this laboratory several years ago by the action of a digested aqueous extract of pancreas on yeast nucleic acid. A provisional announcement had been made[9] that this preparation contained adenine-uracil di-nucleotide. By hydrolysis of the substance with sulfuric acid we obtained about five times as much adenine as guanine. A study of its phosphorus partition[5] gave exact di-nucleotide values. Evidently both the adenine and the guanine are in di-nucleotide combination.

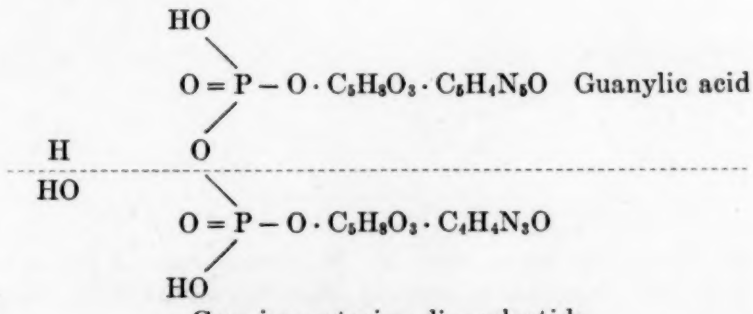
CONCLUSION.

There is no evidence to show that a tri-nucleotide can be produced from yeast nucleic acid either by the action of ferments or by hydrolysis with ammonia. All of the available evidence goes to show that when yeast nucleic acid undergoes hydrolysis, whether by the action of ammonia or under the influence of ferments, the substance is first decomposed into two di-nucleotides, one of which contains the adenine and uracil groups while the other contains the guanine and cytosine groups,



Yeast nucleic acid

The adenine-uracil di-nucleotide is comparatively stable but the guanine-cytosine di-nucleotide easily undergoes hydrolysis into its component mono-nucleotides,



Guanine-cytosine di-nucleotide

This last decomposition occurs to a considerable extent when the substance, *i.e.*, yeast nucleic acid, is heated for an hour or two with 2.5 per cent ammonia at 115° but to a much greater extent when the heating is done at 125°. At 125° the products begin to lose phosphoric acid and nucleosides are formed. At 145° all phosphoric acid is set free and the formation of nucleosides is complete.

NOTE ON HYDROLYSIS OF YEAST NUCLEIC ACID IN THE AUTOCLAVE.

By P. A. LEVENE and W. A. JACOBS.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, April 18, 1916.)

It is customary in this laboratory to heat autoclaves by means of an oil bath, measuring the temperature of the oil bath. In a previous article[1] the temperature referred to was that of the oil bath. This was made clear in the directions given by one of us in a later article.[2] In the latter article it was also stated that a temperature of 135° was sufficient when hydrolysis was carried out in a sealed tube. Consequently the results of Walter Jones[3] essentially agree with those obtained by us.

[1] Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, 1910, xliii, 3154.
[2] Levene, *Abderhalden's Handb. biochem. Arbeitsmethoden*, 1911, v, 492.
[3] Jones, W., and Germann, H. C., *J. Biol. Chem.*, 1916, xxv, 93.

THE NATURE OF THE DIETARY DEFICIENCIES OF THE WHEAT EMBRYO.*

By E. V. McCOLLUM, NINA SIMMONDS, and WALTER PITZ.[1]

(From the Laboratory of Agricultural Chemistry of the Wisconsin Experiment Station, Madison.)

(Received for publication, March 25, 1916.)

In former papers from this laboratory we have shown the character of the deficiencies of the wheat kernel[2] and of polished rice[3] respectively as nutrients for growth and reproduction. As a result of this work, together with an extensive experience in observing animals fed with mixtures of purified foodstuffs, we proposed the working hypothesis that in addition to protein, carbohydrate, or fat and mineral elements in suitable amounts and proportions, the diet, in order to support growth or long continued well-being, must contain two factors whose chemical natures are at present unknown. One of these is associated with fats from certain sources, but is not found in the fats of many natural foodstuffs.[4] The other, which is soluble in water and alcohol, is widely distributed in natural foods such as milk, eggs, meats, vegetables, and grains.[3] This factor is apparently never associated with fats. Funk and others have recognized one of these dietary factors, the water-soluble one, in their studies on beri-beri. There is no

* Published with the permission of the Director of the Wisconsin Experiment Station.

[1] Miss Marguerite Davis participated in a considerable amount of preliminary work directed toward the solution of the problems discussed in this paper. Most of the data accumulated in that work are not included in this discussion, but the information gained therefrom made possible the successful planning of the rations later employed. Credit is due Miss Davis for her part in this investigation.

[2] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxi, 615.

[3] McCollum and Davis, *J. Biol. Chem.*, 1915, xxiii, 181.

[4] McCollum and Davis, *J. Biol. Chem.*, 1913, xv, 167; 1915, xxiii, 231. Osborne, T. B., and Mendel, L. B., *ibid.*, 1913–14, xvi, 424.

doubt that a deficiency of this factor and not the fat-soluble unknown is responsible for the onset of polyneuritis and that the pathological symptoms are entirely relieved by treatment of pigeons with extracts of substances which contain the former but do not furnish the fat-soluble one. McCollum and Kennedy[5] have discussed the desirability of discontinuing the use of the term "vitamine" for these substances which are essential components of an adequate diet, and have suggested the employment of the terms fat-soluble A and water-soluble B instead. These terms are employed in this sense in the present paper.

We deem it of the greatest importance for the purpose of placing human nutrition and animal production upon a sound scientific basis to have, in addition to a full understanding of the essential factors in the diet for growth and the promotion of well-being during maintenance, a thorough knowledge of the causes which are responsible for the failure of nutrition of an animal when confined to a single naturally occurring food substance. We should understand fully why, for example, the corn, wheat, oat, or rice kernel is, individually, totally inadequate to support the life of a grown animal over more than a brief period, or to support growth in the young, as our experience has shown to be the case. Only then shall we be able to proceed to an experimental inquiry into the reasons for the nutritive failure of human beings when eating a liberal variety of foods of vegetable origin, as Goldberger has shown to be the case among pellagrins,[6] or of animals fed certain mixtures with the customary complexity of animal husbandry practice.[7] It is with a view to the acquisition of such knowledge that we are pursuing studies of the character reported in this and the previously cited papers.

The method pursued in our work is based on the following line of reasoning: If a single natural food product fails to nourish an animal adequately, it may be due to: (*a*) lack of sufficient protein, or to proteins of poor quality; (*b*) an unsatisfactory mineral content due either to inadequacy of certain elements in amount, or to unsatisfactory proportions among them; (*c*) an inadequate sup-

[5] McCollum, E. V., and Kennedy, C., *J. Biol. Chem.*, 1916, xxiv, 491.

[6] Goldberger, J., *J. Am. Med. Assn.*, 1916, lxvi, 471.

[7] Hart, E. B., McCollum E. V., Steenbock, H., and Humphrey, G. C., *Wisconsin Agric. Experiment Station, Research Bull. 17*, 1911.

ply of the fat-soluble A; (*d*) of the water-soluble B; (*e*) or some toxic substance contained therein. One, two, three, four, or all of these factors may operate in inducing nutritive disturbances.

It should be obvious that a systematic procedure in which we feed the substance under investigation supplemented with (*a*) pure protein only, (*b*) salt mixture additions only, (*c*) butter fat only, (*d*) extracts known to carry the water-soluble B and as little else as is possible, will reveal whether the failure of nutrition involves one factor only, or more than one. If more than one factor is involved, a similar procedure, but with the addition of all possible combinations of pairs of the isolated food ingredients listed above, followed if need be by another series of feeding experiments in which animals are fed the natural foodstuff supplemented with three such uncomplicated additions, in all possible combinations, and if necessary another experiment in which all four additions are made, will give us results which make it possible to consider the components of our rations in an entirely new light. Provided the foodstuffs contain a toxic substance, special procedures will have to be devised for studying its effects.

Similar studies must also be made by this method of procedure, with pairs of the important foodstuffs in varying proportions, the variation of the mixture including sufficient range to reveal the degree to which the deficiencies of the protein mixture of one grain are corrected by the peculiar quantitative relationships among the amino-acids yielded by the proteins of the other grain. The same may be said for the factors other than protein. In this way we shall become able to interpret the biological value of the mixtures of natural foodstuffs which make up the rations which are in common use, in which the attempt is now made to make for safety through variety. We have carried our inquiry into the nature of the dietary deficiencies of several natural products far enough to convince us of the practicability of this method of study. Young rats fed wheat embryo alone never make any growth, although they may live somewhat beyond 4 months (Chart I). When young rats are fed a mixture of 60 parts of wheat embryo and 40 of dextrin, a mixture which furnishes about 20 per cent of protein, very slow growth takes place, and the animals may live for 4 months or more without loss of weight (Chart II). The addition of calcium lactate (2 per cent) to this food mixture will

at any period of their growth suspension induce a noticeable increase in body weight.

We were led to conclude by our experiments on growth in animals fed diets of low protein content, and by metabolism studies with pigs which received all their protein from the wheat embryo, that these proteins have a high biological value. Rats can make an appreciable amount of growth on diets carrying but 4 per cent of wheat embryo proteins,[8] and pigs can retain as much as 39 per cent of the ingested wheat embryo proteins for growth.[9] Since it is not possible when 60 per cent of wheat embryo is employed in the ration to imitate by salt additions the mineral content of any rations which we have fed successfully, because of the high mineral content of the embryo itself, we tried lowering the content of this constituent to give a protein content of 10 per cent in order to be able to make salt additions to the diet with the prospect of rendering the mineral content a favorable one for growth. Lot 521 (Chart III) shows how marked is the improvement of the animals resulting from the correction of the inorganic content of the diet. After 2 to 3 months failure supervenes, accompanied by what appeared to be an infection of the ears, and an edema of the eyes which is frequently seen in rats suffering from an inadequate supply of the fat-soluble A. Ether-extracted wheat embryo gave us much better nutrition than unextracted material. The cause of this will appear later.

That the failure of nutrition of the rats in Lot 521 (Chart III) was due to a lack of the fat-soluble A is made apparent by the performance of Lot 397 (Chart IV) whose ration was identical with that of Lot 521 except that 5 per cent of butter fat replaced an equivalent amount of dextrin. These rats grew to normal size more rapidly than the curve of normal expectation led us to anticipate and the two females each produced two litters of young. None of these young were reared, however. This and the fact that the three rats in this group all died after 9 and 10 months, led us to suspect that the ration still possessed certain unfavorable factors in some degree. The low protein content may be sufficient to explain this, but the history of Lot 508 (Chart

[8] McCollum and Davis, *J. Biol. Chem.*, 1915, xx, 415.

[9] McCollum, E. V. See Henry, W. A., and Morrison, F. B., Feeds and Feeding, Madison, 15th edition, 1915, 80.

V) points to some other explanation as more probable. It is interesting to note that if Ration 397 is fed without the salt mixture addition it is incapable of inducing any growth whatever (see Chart VIII, Lot 585). The specific reasons for this we shall discuss later in connection with another investigation.

Lot 508 (Chart V) received the same ration as Lot 397 (Chart IV), except that 5 per cent of dextrin was replaced by 5 per cent of purified casein. The growth of these animals has been normal, but two females have each failed to keep their two litters of young alive beyond the first 3 days of life. We were, therefore, led to conclude that there is a slight degree of toxicity in this ration. The protein factor in Lot 397 is therefore not the only factor involved in preventing repetition of reproduction and the successful rearing of the young.

That there is in reality a certain degree of toxic effect of wheat embryo and that this resides in the oil is clearly shown by the records of Lot 495 (Chart VI) whose ration differed from Lot 397 only in that the wheat embryo was not extracted with ether. This group made about half normal growth during 3 to 4 months, as contrasted with a somewhat more rapid increase in weight by Lot 397 than our normal curve predicts. All the animals in Lot 495 became emaciated and presented a very miserable appearance.

The ration of Lot 415 (Chart VII) differed from the preceding one in having 10 per cent more wheat embryo (43 per cent of the food mixture). Although the protein content of this ration was raised to 13 per cent by this modification and was 25 per cent greater than in Lots 397 and 495 (Charts V and VI), there was but slight improvement in the condition of the rats. They presented the same general symptoms as did Lot 495. The removal of the fats by extraction with ether removes in great measure the toxicity of the embryo. We are at present conducting experiments to determine whether the toxicity of these fats resides in the fatty acids themselves or is due to some substance soluble in the fats and in ether.

The marked difference between the nutritive value of the ration of Lot 508 (Chart V) with and without the butter fat addition is illustrated by comparing this group with Lot 566 (Chart IX) which received no butter fat. There was a rapid increase in

weight during the first 4 weeks in some of these rats, but complete failure of nutrition was never deferred much beyond 70 days, and in some cases it took place much earlier. The fat-soluble A must be added to wheat embryo, regardless of what other additions or modifications may be made with diets of the character we are here considering before normal nutrition can be attained. This does not imply the absence of this factor from the wheat embryo; indeed McCollum and Davis[10] have demonstrated that the latter does contain it, and this is further supported by the records of Lot 498 (Chart X). This ration will not induce growth without the addition of the fat-soluble A. 5 per cent of ether-extracted wheat embryo carries enough of this substance to induce growth for 90 days in a manner similar to that induced by carefully purified protein, carbohydrate, and salts, with a content of 20 per cent of milk sugar of ordinary laboratory purity. Such lactose carries a sufficient amount of both the fat-soluble A and the water-soluble B to induce growth in vigorous individuals at but little below the average normal rate, over periods of 60 to 100 days.[11] This result reveals the unexpected fact that in removing the fats from wheat embryo by extraction with ether the fat-soluble A is not wholly extracted. Lot 509 (Chart XI) further illustrates this fact. This ration, without the factor in question, does not support growth. Both the unknown A and B are supplied by 5 per cent of fat-free germ, in amounts sufficient to induce good growth over several months. It is this tendency of the fat-soluble A to remain associated with lactose, together with the generous amount of the water-soluble B contained in milk serum which determines the growth-promoting power of Osborne and Mendel's protein-free milk. Provided these factors are added, success can be attained in nourishing growing animals with salt mixtures prepared from purified reagents. Since we have shown how the water-soluble B can be obtained from various sources by the use of several solvents, it is now easy to conduct experiments to compare the relative values of purified proteins under experimental conditions much more satisfactory than to employ purified proteins in rations containing 6.9 per cent of their total nitrogen in unknown forms, as was done by Osborne and Mendel

[10] McCollum and Davis, *J. Biol. Chem.*, 1915, xxi, 179.

[11] McCollum and Davis, *J. Biol. Chem.*, 1915, xxiii, 231.

in all cases where the diets contained 18 per cent of protein and 28.3 per cent of protein-free milk which contained 0.76 per cent of N. The desirability of improving the technique of this line of investigation is emphasized by our observations that the nitrogen of protein-free milk appears to possess considerable value as a source of protein cleavage products.[8]

That it is not lack of fats which caused the nutritive depression in Lot 566 (Chart IX) is made apparent by the records of Lot 523 (Chart XII). These rats were given a ration precisely like Lot 508 (Chart V) except that 5 per cent of corn oil replaced 5 per cent of butter fat. During 98 days these animals were markedly depressed in growth, the depression manifesting itself only after 30 days. After 98 days butter fat was added to the diet, with the result that growth was at once resumed. The marked inferiority of corn oil with respect to its content of the fat-soluble A as compared with an equivalent amount of butter fat is emphasized by these results. That corn oil is not entirely without this growth-promoting factor is indicated by unpublished results of this laboratory.

Although in Lot 397 (Chart IV) 10 per cent of wheat embryo proteins sufficed for normal growth and supported females while they produced two litters of young (but without rearing the young), and the ration was not improved so far as the rearing of the young was concerned by the addition of 5 per cent of casein (Lot 508, Chart V), the food mixture is rendered more satisfactory for resistance to unfavorable factors which may operate in the diet. Thus in Lot 520 (Chart XIII) the ration resembled that of Lot 523 (Chart XII) in all respects except that the 5 per cent of casein of the latter's ration was replaced by dextrin. These animals receiving corn oil as their only source of fat did not make as good growth without the casein added (Lot 520) as did Lot 523.

This case illustrates a principle in nutrition which has not hitherto been fully appreciated; *viz.*, that a single factor (protein) in a ration may appear to admit of the maximum performance of the animal with respect to growth, without itself representing the optimum amount or character. When this circumstance prevails, it may entirely escape notice, yet if in another ration exactly like it except that a second factor tends to injure the animal, nutritive failure may result. In such a case as the latter, the im-

provement of the protein factor by the addition of more protein or by the substitution of a better protein, the plane of protein intake remaining unchanged, the animal may make the maximum performance notwithstanding the unfavorable character of the injurious factor of the ration.

For example, Lot 397 (Chart IV) contains but 10 per cent of protein, all furnished by ether-extracted wheat embryo. This suffices to support growth at the maximum rate. In Lot 495 (Chart VI), whose ration was exactly similar except that the wheat embryo carried the toxic wheat embryo fats, marked depression of growth was observed. Lot 524 (Chart XIV) received the same ration as did Lot 495, but with 5 per cent more protein in the form of casein, replacing dextrin, and in spite of the toxic effect of the embryo fats growth was normal for a period of over 4 months and three females produced six litters of young during that period, although none of these were reared. This additional 5 per cent of casein caused Lot 524 to overcome the toxic action of the fats in a considerable degree. Lot 405 (Chart XV), whose ration differed from Lot 524 only in having but 2 per cent of butter fat while the latter had 5 per cent, illustrates the same principle. With the low butter fat content the casein content like that of Ration 524 did not protect the animals from the stunting effect of the toxic fats. The 5 per cent of casein together with the higher content of butter fat served to make the rats of Lot 524 more resistant to this unfavorable factor. Such results suggest a plausible explanation for the marked difference in the resistance to poisoning with acetonitrile, shown by rats and mice, which resulted in the experiments of Hunt,[12] through the influence of the diet. We have elsewhere called attention to this same principle in its operation in determining whether the inorganic content of the diet, when of unsatisfactory make-up, will produce noticeable depression in the growth and health of the experimental animals.[13]

Further illustration of the toxic effect of wheat embryo fats is seen in Lot 517 (Chart XVI) whose ration contained but a trace of fats from this source, and Lot 448 (Chart XVII) whose ration was exactly similar except that the 6 per cent of dextrin in the former was replaced by 6 per cent of wheat embryo fats in

[12] Hunt, R., *Bull. Hyg. Lab., U. S. P. H., 69*, 1910.

[13] McCollum and Davis, *J. Biol. Chem.*, 1915, xxi, 641.

the latter. The growth curves of Lot 448 are depressed in an easily noticeable degree, and a tendency to loss of hair in spots further points to the unfavorable effects of this addition.

The question naturally arises on inspecting the data presented in this paper, when in Lots 498 and 509 (Charts X and XI) 5 per cent of ether-extracted wheat embryo served to furnish enough of the fat-soluble A to induce nearly normal growth during a period of 3 to 4 months, why does not a ration carrying 33 per cent of wheat embryo supply an amount of this substance which meets all the needs of the animals? In all such rations we see failure without butter fat (Lots 520, 521, and 523) and pronounced benefit from its addition to or inclusion in the diet (compare Lot 521, Chart III, with Lot 397, Chart IV; Lot 566, Chart IX, with Lot 508, Chart V; Lot 405, Chart XV, with Lot 524, Chart XIV). This question seems to admit of but one explanation in the light of the data available, and this we have adopted tentatively: Wheat embryo is not entirely freed from its toxicity even by long continued extraction with ether (20 hours). When therefore the extracted material is added in progressively greater amounts in a series of rations two factors are affected which operate in opposition to each other physiologically. When a low plane of fat-free embryo is employed a small amount of fat-soluble A is introduced, and but little of the toxic substance. The amount of the fat-soluble A is not great enough to support young rats from infancy through complete growth, and failure ultimately supervenes. The toxic factor may even here operate in some degree to bring about nutritive failure, through a cumulative effect. When higher planes of ether-extracted wheat embryo are employed, the injurious effects of its toxicity tend to counterbalance the beneficial effect of the added fat-soluble A, and the animal is prevented from developing normally.

This hypothesis is in some measure supported by the behavior of Lot 463 (Chart XVIII) whose ration consisted of ether-extracted wheat embryo 60.0, salts (314) 5.0, and dextrin 35.0 per cent. These rats grew at about one-third the normal rate during the first 100 days. Thereafter two of the four made a decided response in growth when butter fat was added to the diet. It is recognized that the mineral content of this ration is not satisfactory for growth when any other factors are unfavorable.

We present also the records of Lot 463-A (Chart XIX) whose ration consisted, aside from its content of 2 per cent of wheat embryo, of substances free from the water-soluble B. Even this small amount of embryo supplied this factor in quantity sufficient to promote growth and well-being over a long period. The content of water-soluble B in this ration is doubtless near the minimum on which nutrition can remain nearly normal, and it is highly probable that the resistance of these rats to unfavorable agencies of any character would be heightened by a higher content of the water-soluble B in the diet.

In former papers from this laboratory we have pointed out the distinctly injurious effects resulting from feeding rations too closely restricted to the wheat plant and its parts.[7, 14] The necessary modifications of the wheat kernel which led to successful nutrition with rations consisting principally of the wheat kernel have been described by McCollum and Davis.[2] In the present discussion of the special properties of wheat embryo we are able to point out definitely that the toxic factor is in the fat fraction. There is a possibility that the failure to attain perfect nutrition with rations consisting of fat-free wheat embryo supplemented with isolated food substances such as protein, salts, and butter fat is to be explained as the result of the failure of ether to remove entirely the toxic substance.

SUMMARY OF RESULTS.

The experiments reported in this paper reveal the fact that the wheat embryo contains qualitatively all the factors essential for the promotion of growth and well-being in an animal, but these are not so proportioned that it can serve as a satisfactory diet without several modifications.

The mineral content must be modified in certain respects before growth can proceed at all.

The character of the proteins is excellent; no other proteins from plant sources which we have studied are superior to them. Rations containing but 10 per cent of these proteins are wholly adequate for growth at the maximum rate.

Both the fat-soluble A and the water-soluble B, the factors which must be supplied by an adequate diet, but whose chemical

[14] Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373.

natures are as yet unknown, are present; the first, in moderate concentration; the second, in very high concentration as measured by the needs of the growing animal. 2 per cent of wheat embryo supplies enough of the water-soluble B to promote growth at the normal rate for several months.

There is contained in the wheat germ a substance which is distinctly toxic to animals. This is in great measure removed by extraction with ether, and is found in the fat fraction. We have not yet determined whether the toxicity is due to peculiarities in the chemical nature of the fats themselves or to something which is associated with the fats.

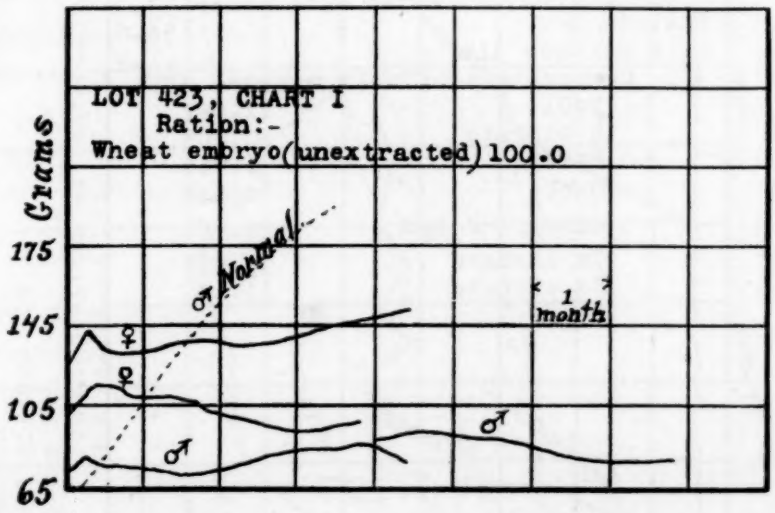


CHART I. Lot 423. These records show the failure of young rats to grow when limited to wheat embryo as their sole source of nutriment. The wheat embryo employed in these experiments contained 30.0 per cent of protein.

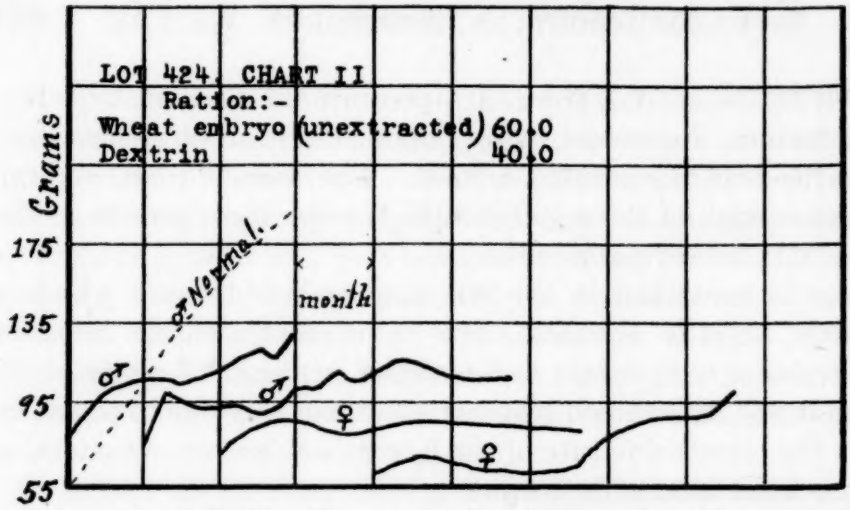


CHART II. Lot 424. Shows a marked improvement in young rats fed wheat embryo 60 and dextrin 40 per cent. With this food mixture slow but long continued growth can take place (compare Chart I).

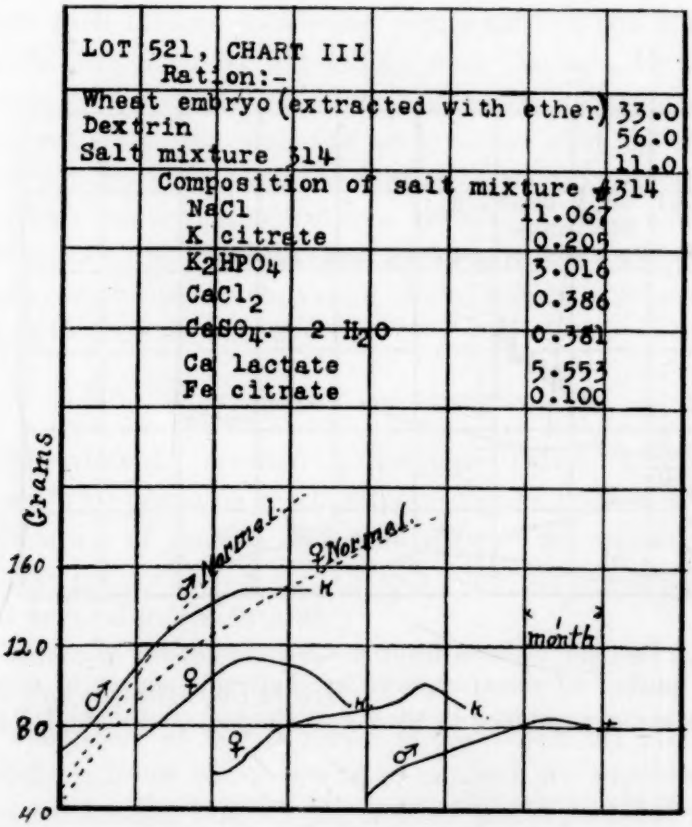


CHART III. Lot 521. Illustrates the marked improvement in the growth of young rats fed ether-extracted wheat embryo to supply 10 per cent of protein together with dextrin and a salt mixture. While failure of nutrition supervenes after 3 or 4 months on this ration, the addition of butter fat renders it surprisingly efficient in promoting growth and sustained well-being (compare with Lot 397, Chart IV).

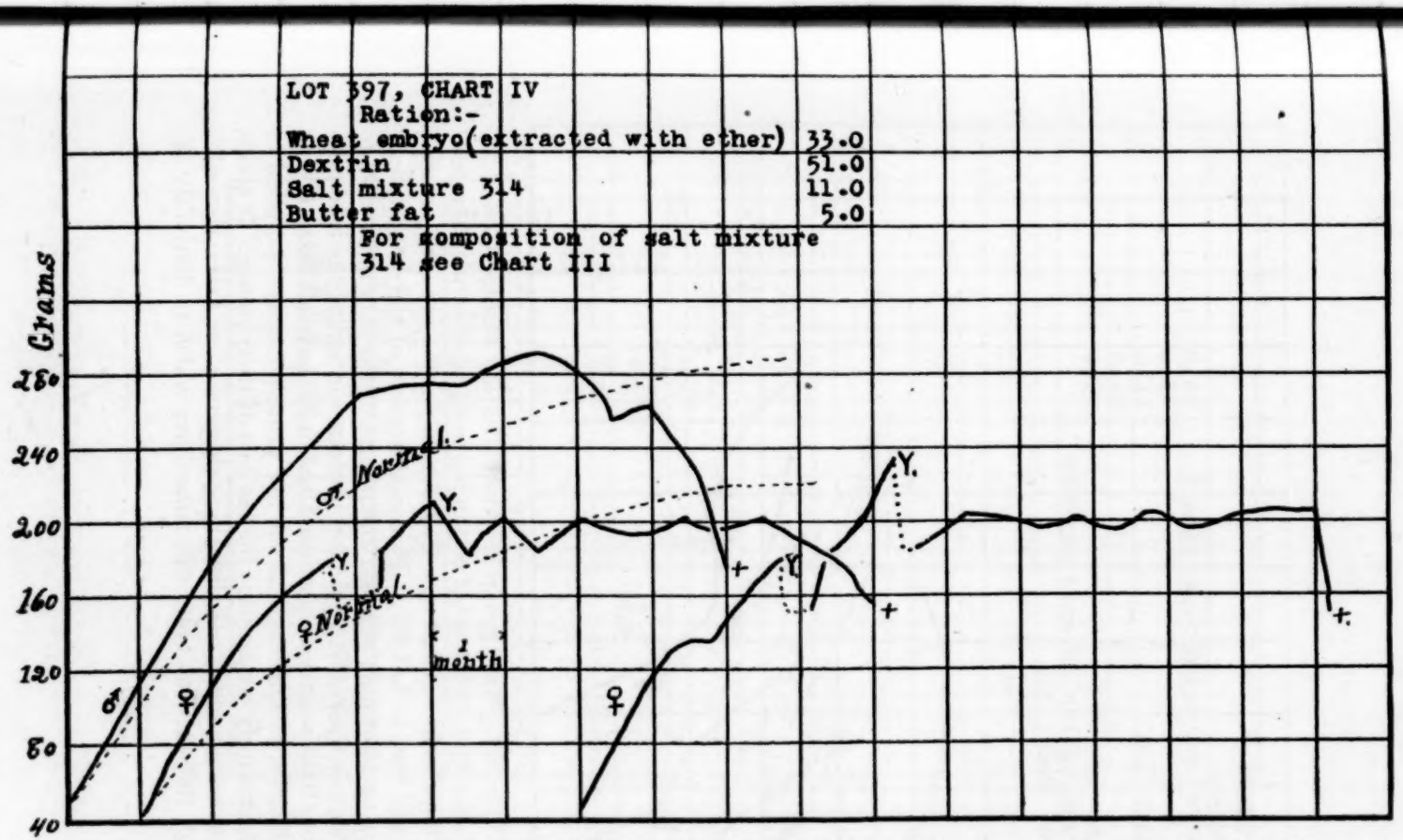


CHART IV. Lot 397. Without the butter fat contained in the food mixture supplied to these rats, depressed growth and early failure supervenes (Lot 521, Chart III). This furnishes the fat-soluble A, a substance of unknown chemical nature which is indispensable for growth.

Wheat embryo fats contain something which is distinctly toxic to animals. This ration if made up with embryo from which the fats have not been removed is distinctly inferior to the above where fat-free embryo was employed (compare Lot 495, Chart VI).

Without the salt mixture additions this ration does not induce any growth (see Lot 585, Chart VIII).

The failure of these rats to produce more than two litters of young each, and their inability to nourish those which they produced, points to the existence of a toxic factor in the wheat embryo after it has been thoroughly extracted with ether. In this and succeeding charts Y marks the birth of young.

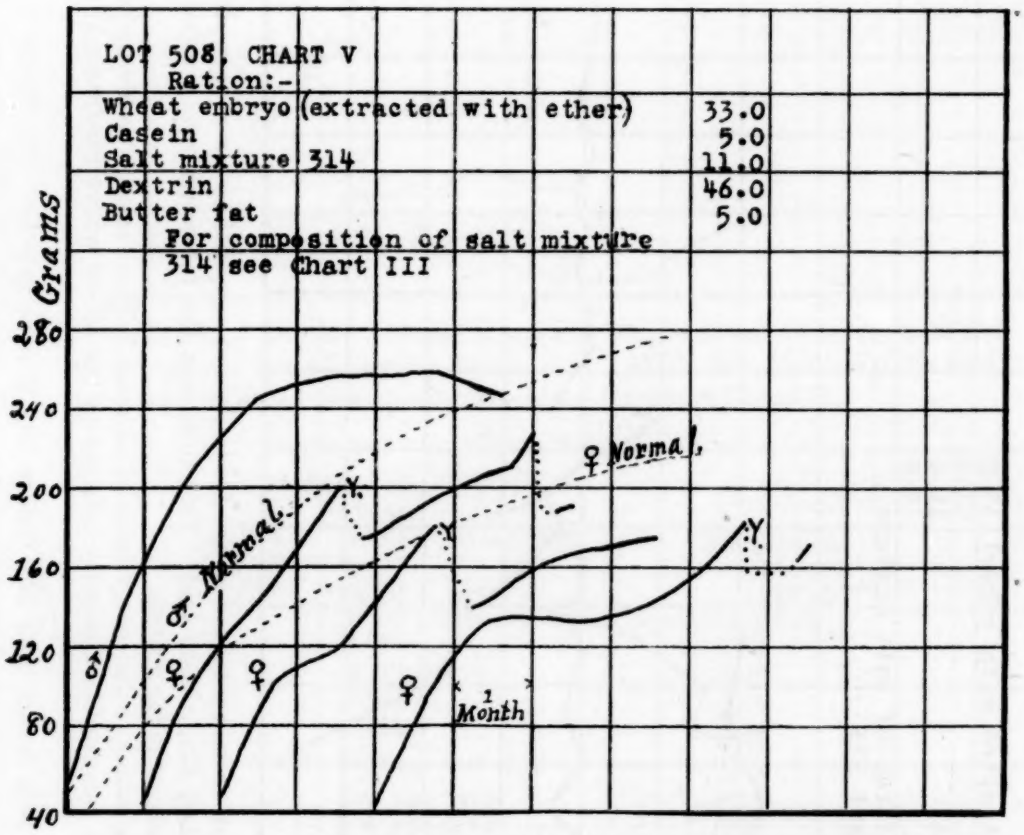


CHART V. Lot 508. This group received a ration similar to Lot 397 (Chart IV), except that 5 per cent of casein replaced an equivalent amount of dextrin. Its protein content was therefore about 15 per cent as compared with 10 per cent in Lot 397. This modification of the diet was employed in order to determine whether the failure of the rats in Lot 397 to rear their young was the result of the low protein content of the food mixture. Since four litters of young produced by Lot 508 have all been allowed to die within 3 days, it appears that the low protein content supplied to Lot 397 was not entirely responsible for the loss of their young. It seems evident that the wheat embryo is slightly toxic even after extraction with ether, although this solvent takes out something which is distinctly injurious.

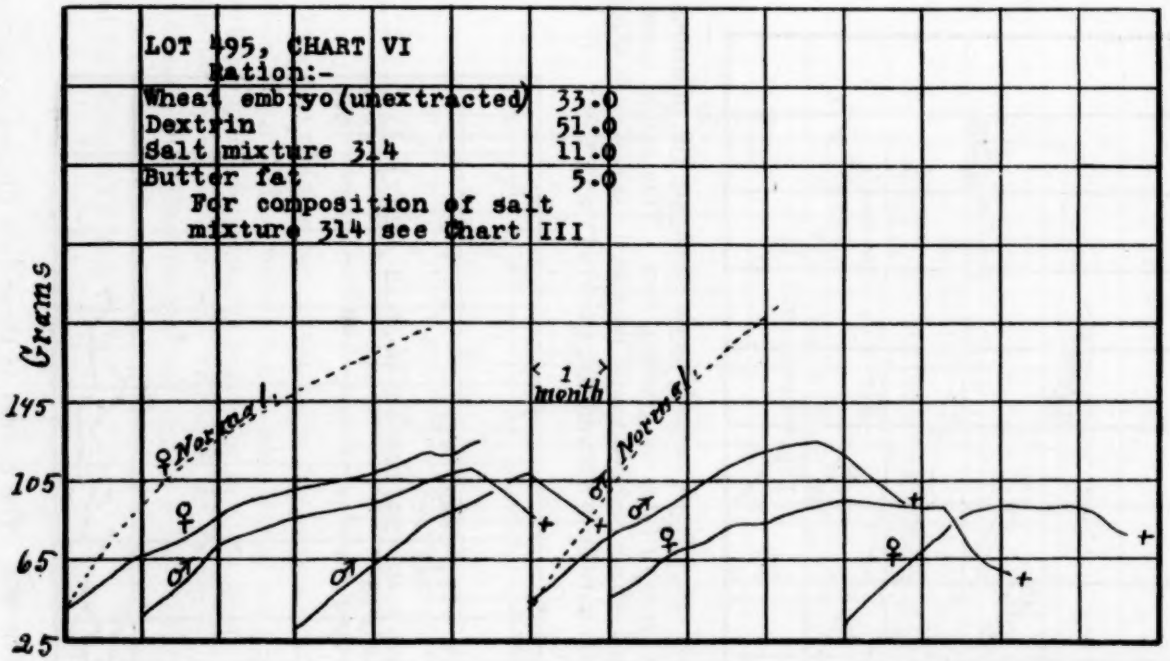


CHART VI. Lot 495. These records show the great inferiority of wheat embryo containing its fat content to that which has been extracted with ether (see Lots 397 and 508, Charts IV and V). The ration here employed differed from that of Lot 397 only in respect to its content of wheat embryo fats. About 3.3 per cent of these fats were supplied by this ration and the rate of growth was depressed about one-half by their presence.

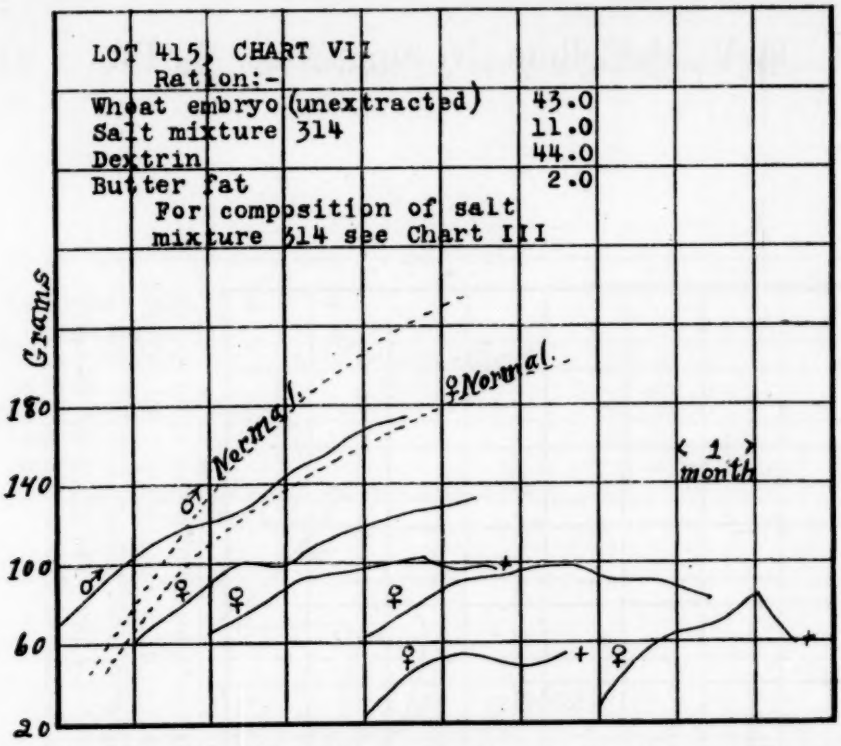


CHART VII. Lot 415. These records again illustrate the injurious effects of the wheat embryo fats, on growing rats. This ration made up with ether-extracted wheat embryo would be greatly superior to the formula here given (compare Lots 397 and 508, Charts IV and V).

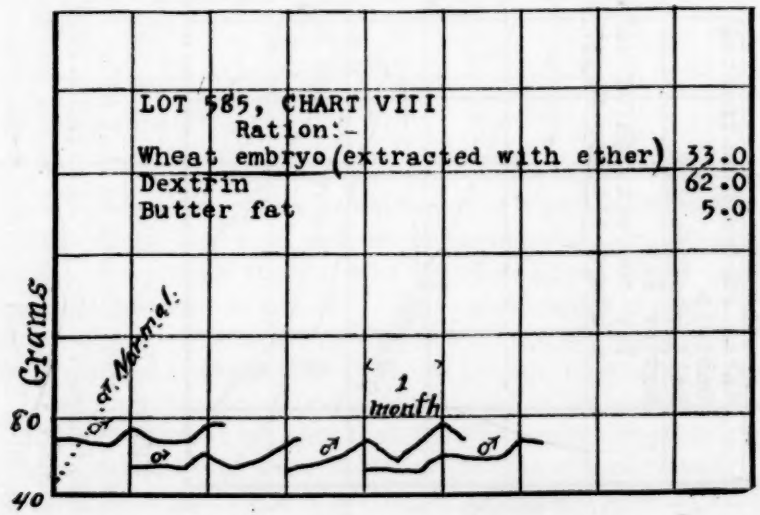


CHART VIII. Lot 585. The curves of these rats illustrate the great importance of the proper adjustment of the mineral content of the diet. The animals in Chart VIII received a ration like that of Lot 397 (Chart IV), except that no salt addition was made, dextrin being added in place of the salt mixture. In this instance we have an example of a ration in which the adjustment of the inorganic content determines whether there shall be complete suspension of growth (without salt additions), or growth at a rate exceeding the normal expectation, as illustrated by Lot 397 (Chart IV).

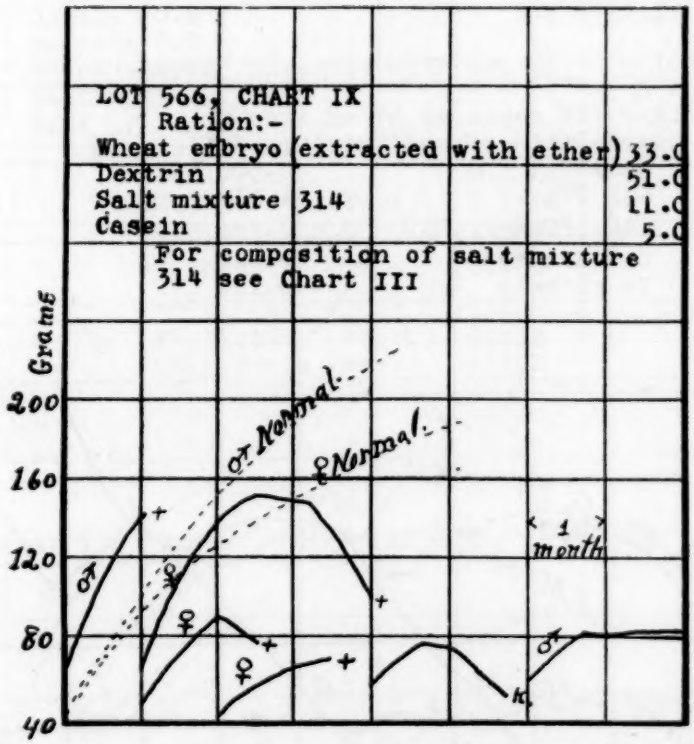


CHART IX. Lot 566. This chart illustrates the great benefit to the animals of including butter fat, or some fat of the group which possesses the same growth-promoting property, in diets where the fat-soluble A is supplied by wheat embryo. Although this factor is present, a higher content makes the difference between almost complete failure and nearly complete success in the nutrition of growing animals (compare with Lots 397 and 508, Charts IV and V).

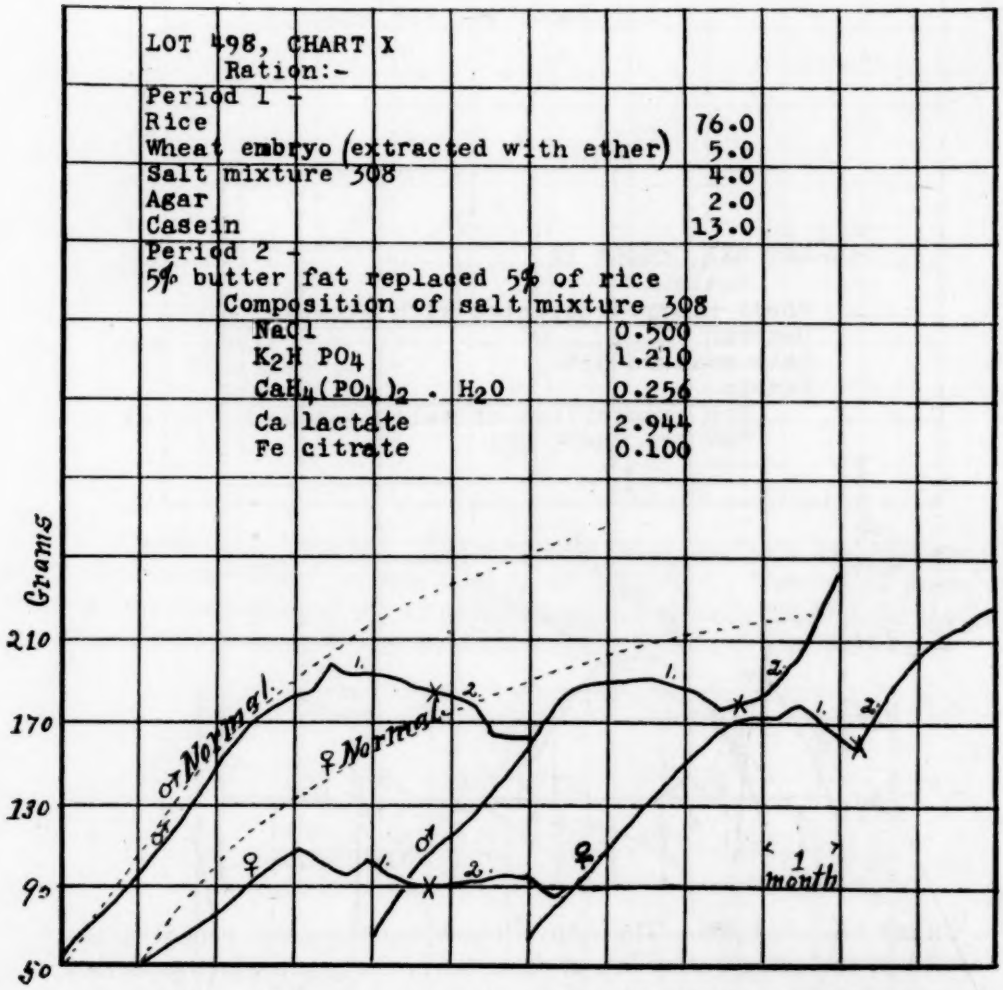


CHART X. · Lot 498. The ration fed this lot will not support growth when either the fat-soluble A or the water-soluble B is absent. The inclusion of 5 per cent of wheat embryo in the food mixture supplies an abundance of the factor B (see Lot 463-A, Chart XIX), and enough of the factor A to induce nearly normal growth in rats over a period of 90 days. The amount is, however, too small to keep the animals in health and growth for a longer period. These results justify the assertion that the fat-soluble A is present in the wheat germ, and confirms our earlier observation on this point.[10]

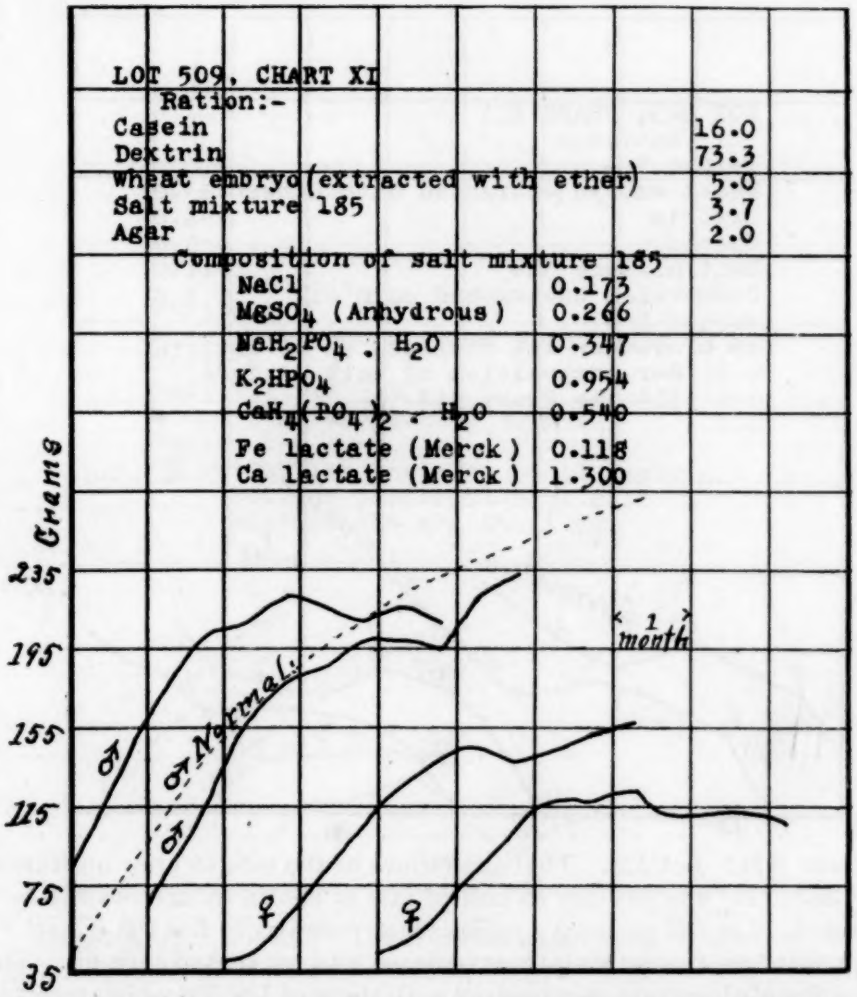


CHART XI. Lot 509. Further illustrates the presence of the fat-soluble A in wheat embryo. Without this factor there would be no growth with this ration. Both the fat-soluble A and water-soluble B are furnished in this ration by 5 per cent of wheat embryo which has been freed from fats by extraction with ether (compare with Lot 498, Chart X, and with Lots 523 and 520, Charts XII and XIII).

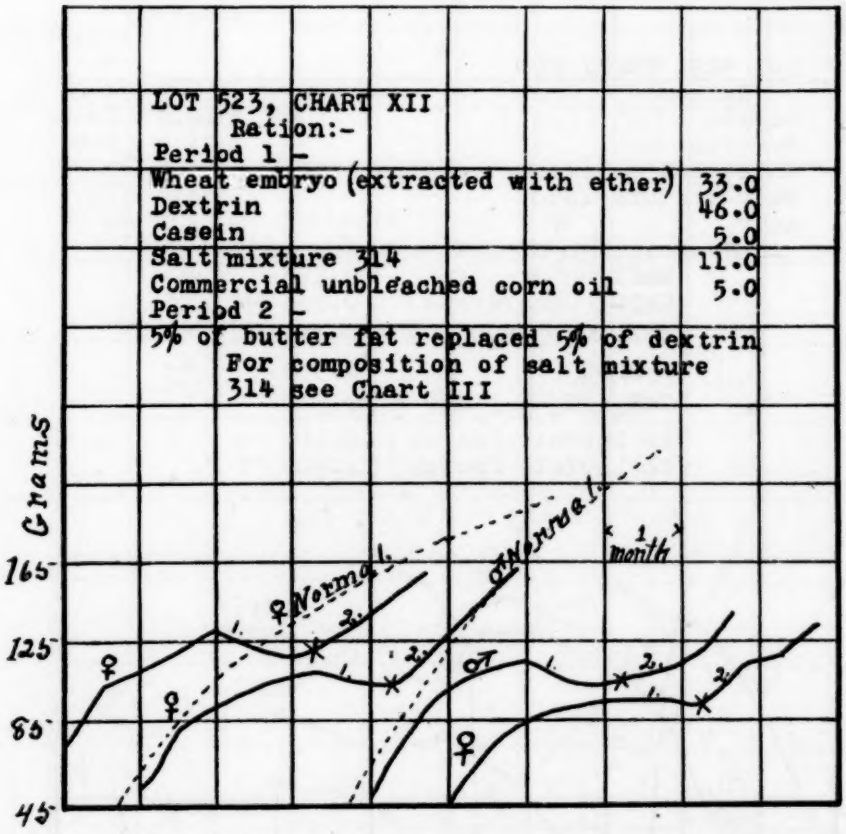


CHART XII. Lot 523. That the failure of the rats to grow on Ration 521 (Chart III) was not due to lack of fats is shown by the records here presented. Lot 523 received a ration closely similar to Lot 508 (Chart V) except that here the butter fat was replaced by 5 per cent of corn oil. The inferiority of these rats as compared with those of Lot 508 which received 5 per cent of butter fat is unmistakable (compare Lot 508, Chart V, and Lot 520, Chart XIII). During the last month covered by these curves 5 per cent of butter fat was included in the ration, and this resulted in a marked response with growth. Corn oil is not the equivalent of butter fat in supplying the fat-soluble A (compare Lot 523 with Lot 508, Chart V, and Lot 397, Chart IV with Lot 520, Chart XIII).

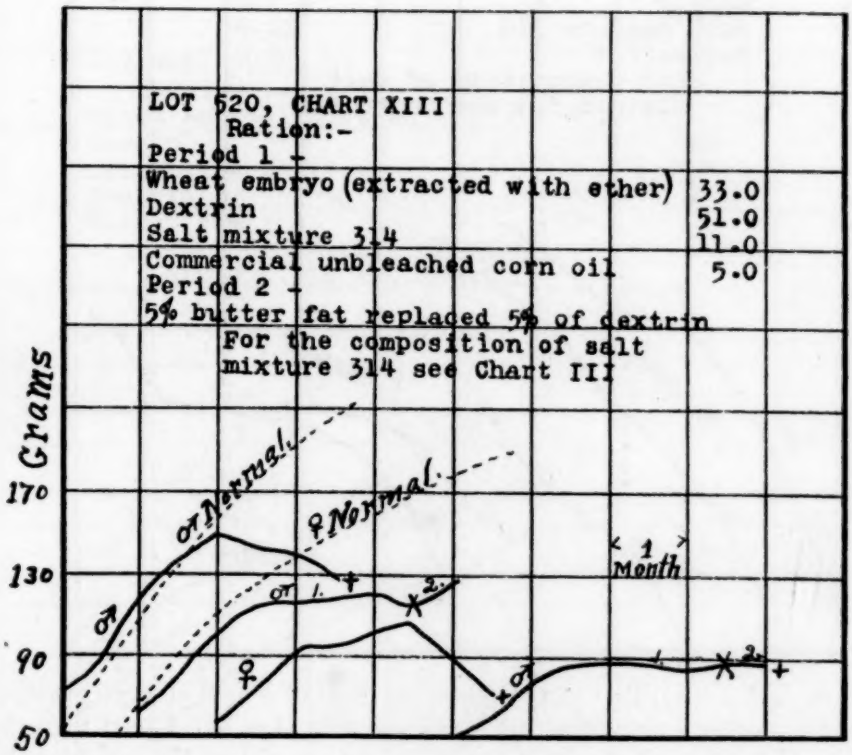


CHART XIII. Lot 520. These curves again point to the fact that corn oil is not equivalent to butter fat in its growth-promoting power. The fat-soluble A is present in the corn kernel, but in relatively low amount (compare Lot 397, Chart IV, and Lot 523, Chart XII).

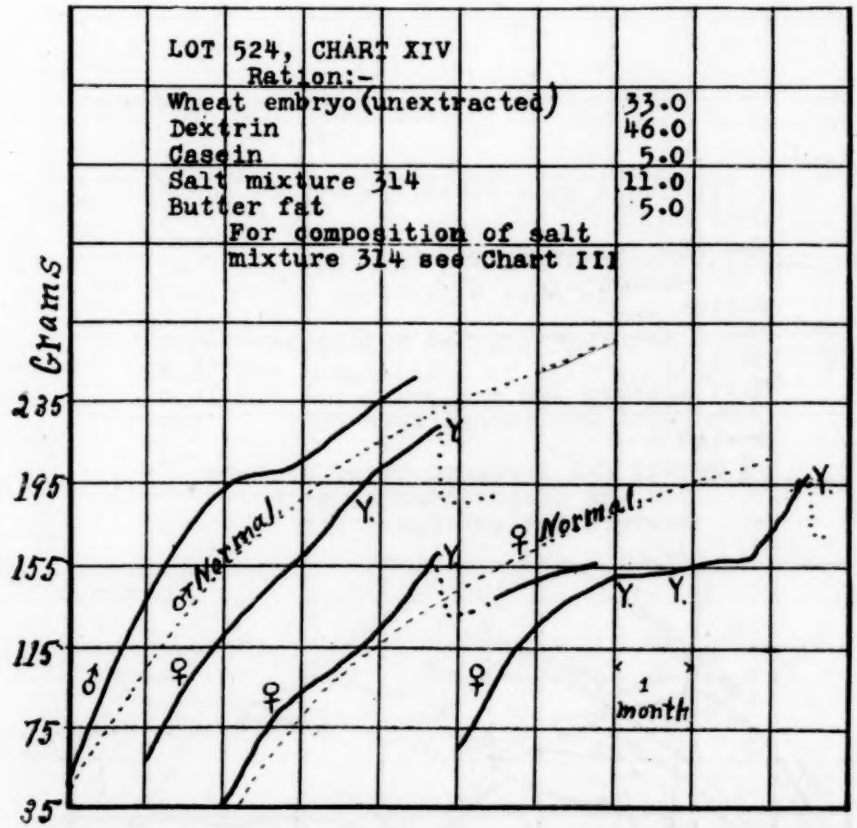


CHART XIV. Lot 524. Although 10 per cent of wheat embryo proteins are able to support growth at the maximum rate (Lot 397, Chart IV), the addition of 5 per cent of casein, as in the ration of Lot 524, enables the animals to withstand to a marked degree the injurious effects of wheat embryo fats (compare Lot 495, Chart VI). The ration of Lot 495 differed from that of Lot 524 only in having 5 per cent of casein replacing dextrin. These records illustrate how an unfavorable factor of any character in the diet may escape notice if all other factors are made favorable. With the protein content less favorable (Lot 495, Chart VI), or with the fat-soluble A reduced to nearly the minimum requirement (Lots 523 and 520, Charts XII and XIII), the toxic effects of the wheat embryo fats manifest themselves.

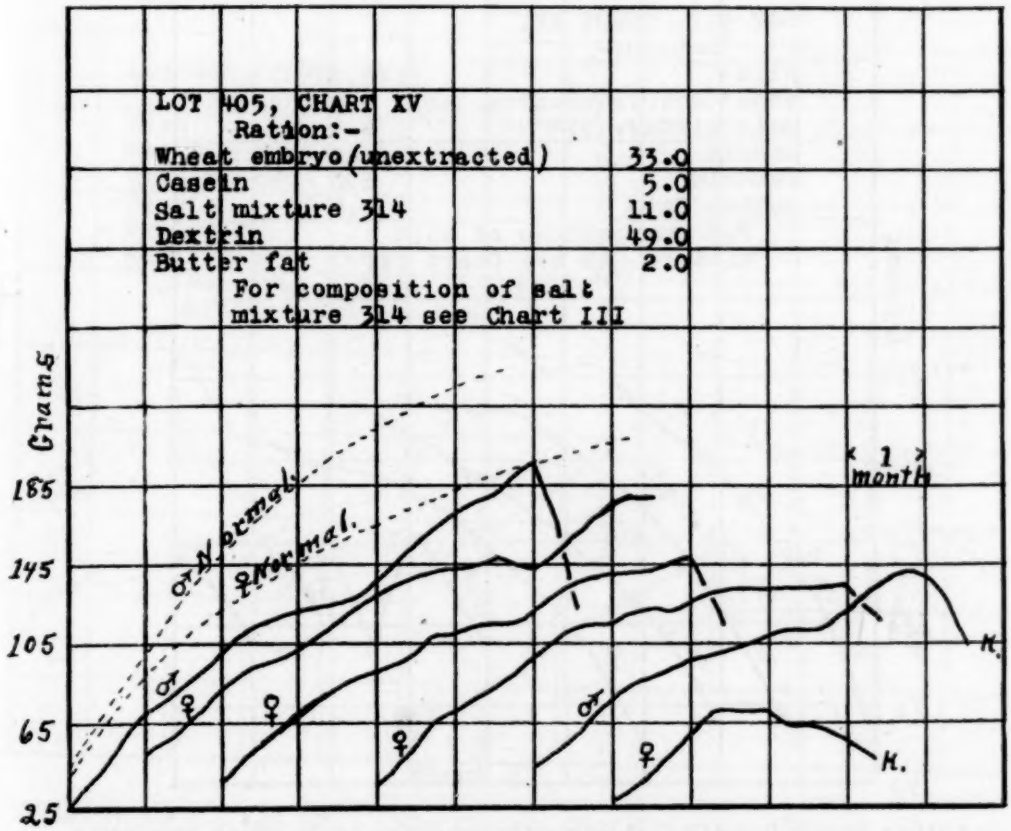


CHART XV. Lot 405. Illustrates the distinct advantage to the growing young of having an abundance of the fat-soluble A in the diet. The curves of Lot 405 which received 2 per cent of butter fat are decidedly inferior to those of Lot 524 (Chart XIV), whose ration differed only in having 5 per cent of butter fat.

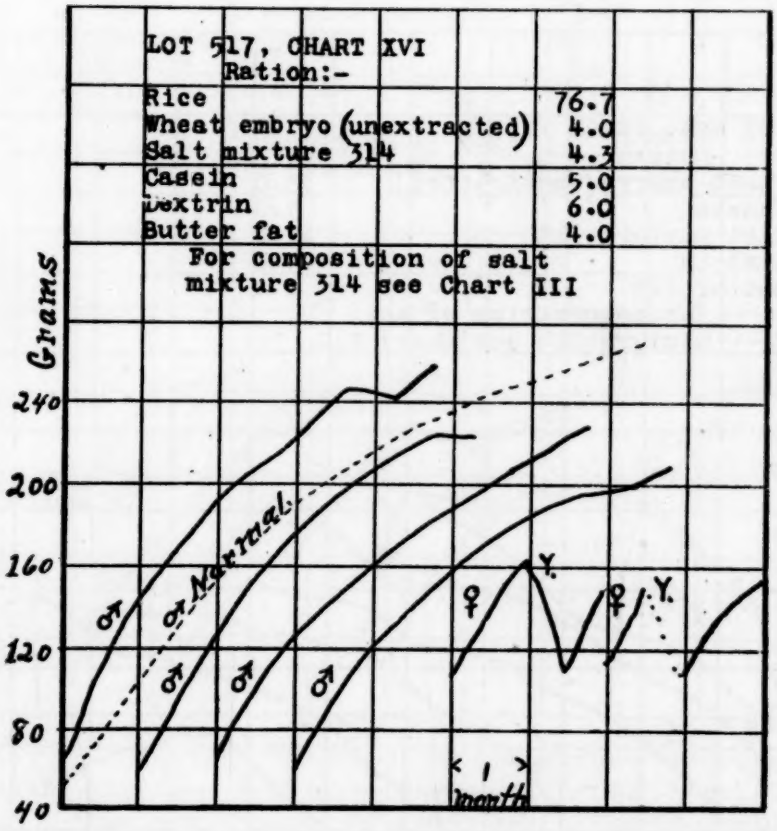


CHART XVI. Lot 517. Showing the records of rats which received a ration containing but a trace of wheat embryo fats, as compared with Lot 448 (Chart XVII), whose ration was closely similar but contained 6 per cent of wheat embryo fats replacing 6 per cent of dextrin. The depressing effect of the wheat embryo fats is readily seen (compare Lot 448, Chart XVII).

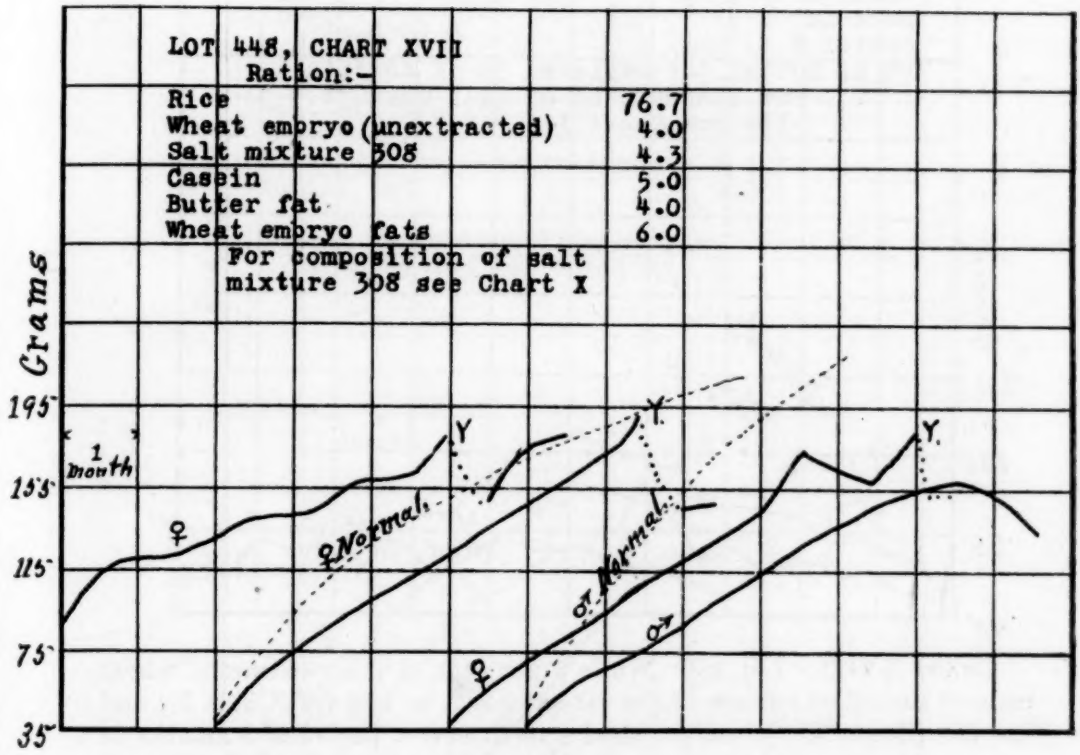


CHART XVII. Lot 448. These records show the toxic effect of 6 per cent of wheat embryo fats in the diet of these rats. Their growth was distinctly below the normal rate, and they were inferior to those of Lot 517 (Chart XVI), whose ration was similar except that 6 per cent of dextrin replaced the wheat embryo fats of Lot 448 (compare Lot 517, Chart XVI).

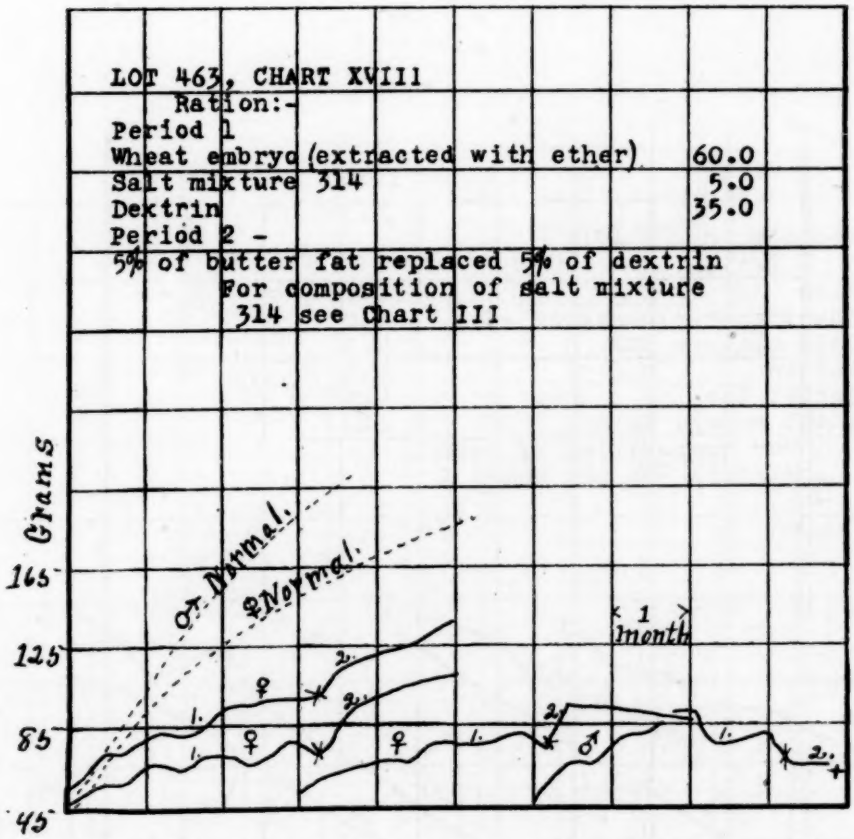


CHART XVIII. Lot 463. While 5 per cent of ether-extracted wheat embryo furnished enough of the fat-soluble A in Lot 498 (Chart X) and Lot 509 (Chart XI) to induce good growth over a period of 3 months or more, animals whose diets contained liberal amounts of ether-extracted embryo (33 per cent or higher) still responded with better growth and improved well-being to the addition of butter fat, which supplies this factor. This we can explain only by assuming that the fat-free embryo still contains something toxic, and that by making all factors except this toxicity as nearly ideal as possible the resistance to the toxic effect is heightened. Lot 463 contained twelve times as much of the fat-soluble A as did Ration 498 or 509, yet growth was depressed and after 100 days the rats still responded in two cases to a butter fat addition. The injurious effect of the toxic factor runs counter to the beneficial effects of the higher level of the fat-soluble A.

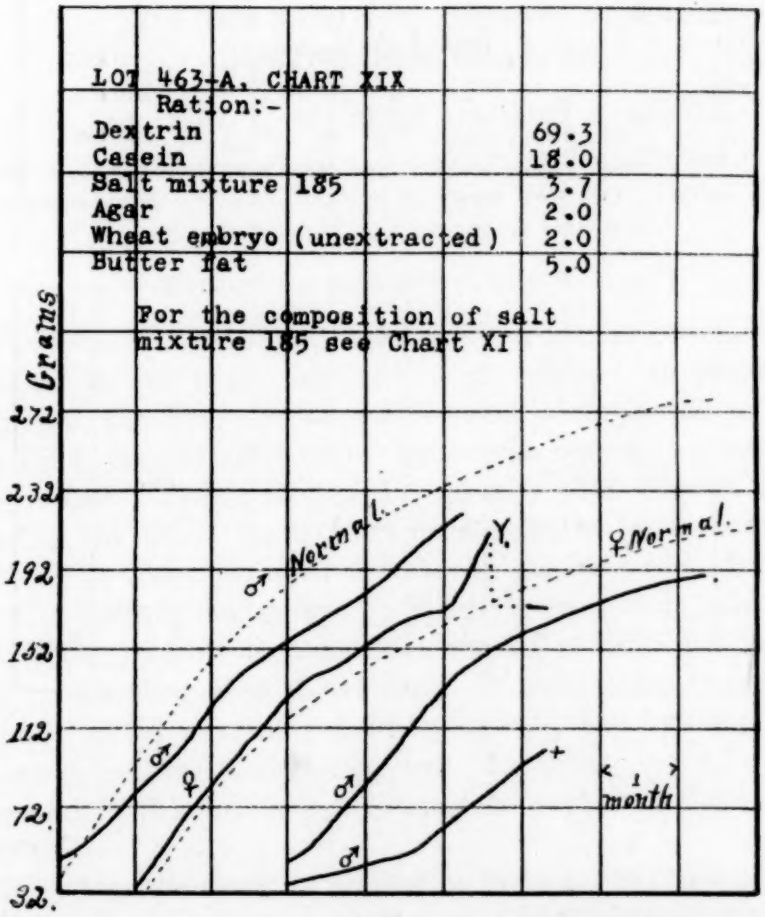


CHART XIX. Lot 463-A. These records illustrate the high content of the water-soluble B in wheat embryo. Without this factor in the diet this ration will not support growth. As little as 2 per cent of wheat embryo furnishes enough of this substance to promote growth over a long period, but is probably somewhat below the optimum.

THE SEPARATION OF AUTOGENOUS AND ADDED HYDROCYANIC ACID FROM CERTAIN PLANT TISSUES AND ITS DISAPPEARANCE DURING MACERATION.*

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(Received for publication, March 23, 1916.)

Hydrocyanic acid is supposed to occur in plants only in combination. It has been identified in glucosides. In plant tissues, therefore, the hydrocyanic acid in such a compound as a glucoside must first be set free by hydrolysis before this acid can be determined. The hydrolysis has usually been effected either by treating the plant tissue with fixed acid, or by macerating the material in water alone, or in water in presence of added emulsin or other hydrolyzing enzyme. When hydrolysis is performed by fixed acids, hydrolysis and distillation are combined in one operation by distilling with dilute acid. If maceration either with or without added enzyme causes hydrolysis, the hydrocyanic acid liberated is subsequently distilled. In either case the hydrocyanic acid in the distillate is estimated by some of the well known methods.

No systematic study of all factors involved in the separation of hydrocyanic acid from plant tissues, such as is customary in the study of analytical methods, has been recorded in the literature. For example, the possibility of loss of hydrocyanic acid during maceration has not been investigated, although there are statements in the literature indicating that such losses occur. Thus, in 1912 Dezani[1] published an investigation showing that cyanide

* The essential facts included in this paper were presented before the Biological Chemistry Section of the American Chemical Society, in session December 27–30, 1911, at Washington, D. C.

[1] Dezani, S., La riduzione dell' acido cianidrico ad opera dei succhi delle piante, *Atti II Cong. naz. chim. appl.*, Turin, 1911, 368.

added to plant juices expressed under a pressure of 300 atmospheres is decomposed with the formation of ammonia. The work reported here by us was essentially completed before Dezani's publication. Further, Waller[2] finds that in the presence of indifferent narcotics, such as ether, chloroform, or alcohol, the rate of evolution of hydrocyanic acid from the leaves of the cherry laurel, *Prunus laurocerasus*, is much less rapid at 16–18°C. than at 40°C. In water at room temperatures practically none is set free. It has usually been assumed that, in distilling in presence of small amounts of the fixed acid, hydrolysis is rapid. Dunstan and Henry[3] have claimed that lotusin, the cyanogenetic glucoside of *Lotus arabicus*, is less rapidly hydrolyzed by sulfuric acid than by hydrochloric acid. Wirth[4] has shown that in distilling the cherry laurel water of the Dutch Pharmacopeia, hydrocyanic acid and aldehyde distill over uncombined, combining, however, in the distillate until a certain equilibrium is reached. Rocques and Lévy[5] have found that in old cherry brandies hydrocyanic acid occurs in combination and is evolved only gradually during distillation. Furthermore, after the conclusion of the experiments recorded in the present paper, De Jong[6] published the observation that the enzyme of *Pangium edule* decomposes the cyanogenetic glucoside, gynocardin, forming both a diketone and hydrocyanic acid, the latter in part disappearing as such because it combines with the diketone. Henry and Auld[7] have shown that to a slight extent sucrose, levulose, and ammonium sulfate retard the rate at which hydrocyanic acid is set free from phaseolunatin by the

[2] Waller, A. D., A new method for the quantitative estimation of hydrocyanic acid in vegetable and animal tissues, *Proc. Roy. Soc., Series B*, 1909–10, lxxxii, 574.

[3] Dunstan, W. R., and Henry, T. A., The nature and origin of the poison of *Lotus Arabicus*, *Phil. Tr. Roy. Soc., Series B*, 1901, cxciv, 515.

[4] Wirth, P. H., Untersuchungen über Blausäure-Benzaldehydlösungen in Verbindung mit Kirschlorbeerwasser, *Pharm. Weekblad*, 1911, xlviii, 1049; cited from *Arch. Pharm.*, 1911, ccxlix, 382.

[5] Rocques, X., and Lévy, L., Sur la nature des composés cyanés des kirschs, *Compt. rend. Acad.*, 1909, cxlviii, 494.

[6] De Jong, A. W. K., La décomposition de la gynocardine par l'enzyme des feuilles de pangium edule, *Rec. trav. chim. Pays-Bas*, 1911, xxx, 220.

[7] Henry, T. A., and Auld, S. J. M., The occurrence of cyanogenetic glucosides in feeding-stuffs, *J. Soc. Chem. Ind.*, 1908, xxvii, 428.

enzyme which accompanies this glucoside in a variety of the lima bean, *Phaseolus lunatus*. Glucose present in small quantities markedly retards the rate at which hydrocyanic acid is set free, while the action of invert sugar is less marked. Apparently, this was ascertained by determining the amount of hydrocyanic acid set free under each set of conditions. The amount of hydrocyanic acid remaining combined seems not to have been determined. Henry and Auld[7] devised a method for the determination of hydrocyanic acid in plant tissues by which the cyanogenetic glucoside is partially separated from the plant tissue by hot alcohol before it is hydrolyzed.

It is, therefore, evident from the literature that the determination of hydrocyanic acid present in plant tissue is a matter of some difficulty. The hydrolysis by means of acid is not applicable in all cases since certain cyanogenetic glucosides, such as amygdalin, under the usual conditions of distillation with acids yield only a small percentage of hydrocyanic acid.[8] The determination of hydrocyanic acid by means of an enzyme may be inaccurate, as pointed out by Henry and Auld, because of the presence of substances such as glucose. Henry and Auld assumed that such substances retard the decomposition of the glucoside by the enzyme. However, they do not state that this phenomenon might not possibly have been due to the combination of the liberated hydrocyanic acid with some constituent of the medium, or to the destruction of the hydrocyanic acid, or to its change in such a way that it is not recoverable during distillation. The possibility that the hydrocyanic acid set free is at once combined or destroyed seems worthy of investigation, especially when the determination is made by macerating plant tissue before distilling. Under these conditions it is probable that the protoplasm of a macerated plant remains active for some time. It is easily possible that this protoplasm retains the power to metabolize the liberated hydrocyanic acid exactly as does the normal plant.

An investigation of the influence of duration of distillation on the yield of hydrocyanic acid from plant tissue and the possibility of the disappearance of hydrocyanic acid during maceration is presented in this paper.

[8] Ludwig, H., Einwirkung verdünnter Säuren auf eine Reihe von Bitterstoffen, *Arch. Pharm.*, 1855, cxxxii, 138.

For this study the cyanogenetic plants *Prunus virginiana* or the common wild or choke cherry, *Andropogon sorghum* or the ordinary sorghum, and the grass *Panicularia nervata*[9] were gathered in the District of Columbia or in Arlington, Virginia, in August and September of 1911 and 1912. For use in control experiments *Sambucus canadensis*, or the common elder, was gathered in 1912. All experiments were performed within 6 hours of the time of collection of the plants. Only the leaves of these plants were distilled from 5 per cent sulfuric acid. The distillate was received in an excess of potassium hydrate. Hydrocyanic acid in the alkaline distillate was determined according to the method devised by Berl and Delpy[10] in which the hydrocyanic acid is converted into Prussian blue and the latter determined colorimetrically.

100 gm. portions of the unmacerated leaves of *Andropogon sorghum*, of *Panicularia nervata*, and of *Prunus virginiana*, and 100 gm. portions of the macerated leaves of *Prunus virginiana* were separately distilled in 5 per cent sulfuric acid, the distillate at the end of each half hour being tested to learn when hydrocyanic acid ceases to be evolved. At a uniform, slow rate of distillation it was found that at the end of the first hour of distilling all of the hydrocyanic acid from *Andropogon* and *Panicularia* had been evolved. It required, however, 4 hours to distill all of the hydrocyanic acid from *Prunus*. This was true of the portions containing the macerated leaves as well as of those containing the unmacerated leaves. Even increasing the rate of distilling to violent boiling did not increase the rate at which hydrocyanic acid was set free.

Experiments were also made which show the effect of maceration on the amount of hydrocyanic acid obtainable from *Prunus virginiana*. Other experiments were made to show the effect of maceration on the amount of hydrocyanic acid recoverable from *Andropogon sorghum* and from *Sambucus canadensis* to which in some cases potassium cyanide alone had been added and to which

[9] Alsberg, C. L., and Black, O. F., Concerning the distribution of cyanogen in grasses, especially in the genera *Panicularia* or *Glyceria* and *Tridens* or *Sieglingia*, *J. Biol. Chem.*, 1915, xxi, 601.

[10] Berl, E., and Delpy, M., Über die quantitative colorimetrische Bestimmungen kleiner Blausäure-Mengen, *Ber. chem. Ges.*, 1910, xliii, 1430.

in others potassium cyanide and glucose had been added. The experiments with *Prunus virginiana* and *Andropogon sorghum* are recorded in the table.

TABLE I.

The Quantities of Hydrocyanic Acid Recoverable from the Leaves of Prunus virginiana and of Andropogon sorghum Macerated with Potassium Cyanide.

No.	Plant.	Weight of leaves.	Volume of 5 per cent H_2SO_4.	Autogenous HCN in leaves estimated as KCN.	Weight of KCN added.	Maceration.		HCN as KCN.		
						Time.	Temperature.	Calculated.	Found.	
		gm.	*cc.*	*gm.*	*gm.*	*hrs.*	*°C.*	*gm.*	*gm.*	*per cent*
				A.						
1	*Prunus virginiana.*	50	350		0.0000	0			0.0545	
2	" "	50	350		0.0000	24	Room.	0.0545	0.0390	71
				B.						
3	*Andropogon sorghum.*	50	300		0.0000	0			0.0234	
4	" "	50	300	0.0234	0.1150	100	Room.	0.1384	0.0931	67
5	" "	50	300	0.0234	0.0575	6	37	0.0809	0.0481	59
6	" "	50	300	0.0234	0.1150	24	37	0.1384	0.0676	49
7	" "	65	150	0.0245	0.0575	18	37	0.0830	0.0585	70
				C.						
8	*Andropogon sorghum.*	50	300		0.0000	0			0.0026	
9	" "	50	300	0.0026	0.1150	18	37	0.1176	0.0650	55

In Experiment 1, given in section A of the table, 50 gm. of the finely comminuted leaves of *Prunus* were distilled at once from 350 cc. of 5 per cent sulfuric acid. In Experiment 2 of the table distillation was made after the comminuted leaves of *Prunus* had macerated in distilled water for 24 hours in a stoppered flask in presence of toluene, enough concentrated sulfuric acid being added just before distillation to make a 5 per cent solution. In other experiments, not tabulated, 50 gm. portions of the comminuted leaves were macerated with 350 cc. of distilled water, or with water and enough toluene to inhibit the growth of microorganisms. After standing 18 to 24 hours enough concentrated sulfuric acid was added to each flask to give a concentration of 5 per cent of sulfuric acid and the mixture was immediately distilled. As appears from section A of the table only a portion of the hydro-

cyanic acid present in the plant was recovered after maceration. In the experiments recorded in section B of the table the same phenomenon in the leaves of *Andropogon sorghum* is further studied. In the case of Experiment 3 the distillation was made at once, as just described for the leaves of *Prunus*. In all of the subsequent experiments the finely comminuted leaves were macerated for varying lengths of time with added potassium cyanide. In Experiment 4 maceration was made at room temperature, while in Experiments 5 to 7 incubation was made at 37°C. For Experiments 8 and 9 of section C of the table 100 gm. of the comminuted leaves of *Andropogon* were heated quickly to the boiling point, and, after cooling, divided into two equal portions. One of these (Experiment 8), after receiving enough sulfuric acid to give a 5 per cent solution, was distilled at once; the other (Experiment 9) was macerated in presence of added potassium cyanide and incubated at 37°C. before distillation from sulfuric acid, as shown in the table. In all cases less hydrocyanic acid was found after maceration, either with or without the addition of cyanide, than could be obtained by distillation of the fresh comminuted leaves. The calculated findings of autogenous hydrocyanic acid are based on the findings given in Experiments 3 and 8.

For purposes of comparison between cyanogenetic and non-cyanogenetic plants, 50 gm. portions of the finely comminuted leaves of *Sambucus canadensis* were distilled at once from 350 cc. of 5 per cent sulfuric acid. No cyanide could be detected in the distillate. Other 50 gm. portions of the leaves of *Sambucus* were allowed to macerate in stoppered flasks for 24 hours in 350 cc. of water in presence of toluene. They were then distilled after adding enough concentrated sulfuric acid to give a 5 per cent solution. No cyanide could be detected in the distillate. To other 50 gm. portions known amounts of a standardized solution of potassium cyanide were added in a total volume of 350 cc. Of these mixtures a number were distilled at once with enough concentrated sulfuric acid to give a 5 per cent solution. All the cyanide added was recovered in the distillate. The other mixtures were not distilled at once, but were allowed to macerate in stoppered flasks in presence of toluene from 6 to 36 hours. All of the cyanide was recovered in these experiments also.

In certain other experiments not recorded in the table 100 gm.

portions of leaves of *Andropogon sorghum* and leaves of *Sambucus canadensis* were macerated with known amounts of added potassium cyanide and 5 gm. of glucose. As controls similar experiments without glucose were performed. After 18 hours of maceration at room temperature, all of the portions were distilled in the manner above described. The amount of hydrocyanic acid recovered was as great from those portions that had been macerated with glucose as from those without it.

It is evident from the experiments reported in section A of the table that the amount of hydrocyanic acid which can be separated by distillation with a 5 per cent sulfuric acid from the leaves of *Prunus virginiana* is greater if they are distilled at once than if they are first macerated. It therefore follows that during maceration the hydrocyanic acid naturally present in the plant is in part either destroyed or converted into a form in which it is not separable by hydrolysis in the usual way. It is evident from experiments recorded in section B of the table that in the case of *Andropogon sorghum* added cyanide can be recovered only in part after maceration. Apparently, the macerating plant tissue exerts the same action upon added cyanide that it exerts upon hydrocyanic acid set free from its glucoside. Results recorded in section C of the table give some indications concerning the nature of the mechanism which is responsible for the disappearance of the cyanide. This experiment shows that in the presence of comminuted sorghum that has been heated, cyanide disappears, at least to as great a degree as in the presence of unheated, comminuted sorghum. It therefore follows that the mechanism is not dependent upon the presence of living protoplasm or of enzymes. Hence it is probable that in the case of sorghum, at any rate, the disappearance of the cyanide is dependent upon some chemical or physical phenomenon. It is probably not due to adsorption by the plant fiber, for, if that were the case, it would have occurred with *Sambucus*. Whether this phenomenon is a reaction with aldehyde or with some other reacting substance, or is some quite different phenomenon remains to be ascertained. It is apparent from the experiments with *Sambucus canadensis* that this phenomenon is not universal in plant leaves. If it is a chemical phenomenon it would be natural to suppose that it might be nitrile formation since most plants contain a variety of aldehydes,

the commonest of which is glucose. That glucose is not concerned in the phenomenon is evident from the experiments above described.

It follows from the results above described, as well as from the statements in the literature, that there are a number of causes for the losses of hydrocyanic acid from plant tissues whereby the accuracy of the determination of hydrocyanic acid in plant tissues is impaired. That these facts must be considered in making such determinations has not hitherto been recognized. Among the causes of these losses are incomplete hydrolysis of the glucoside and disappearance of the hydrocyanic acid during maceration. In making determinations of hydrocyanic acid in plant tissues, therefore, the duration of distillation for the complete removal of the hydrocyanic acid must be determined for each species. Moreover, it is necessary in determining hydrocyanic acid in plants to do so by maceration, by direct distillation, separately, of both the fresh and the macerated plant, and by distillation, after extraction, as suggested by Henry and Auld. In this way the method which yields the greatest quantity of hydrocyanic acid can be determined for each species.

SUMMARY.

In this paper it has been shown that the leaves of *Prunus virginiana* must be distilled with acid 4 hours before all of the hydrocyanic acid is liberated, whereas in *Andropogon* and *Panicularia* less than 1 hour is sufficient to liberate all hydrocyanic acid present. It is further shown that in macerating plant tissues that contain hydrocyanic acid a certain amount of the hydrocyanic acid present or of cyanide that may be added is so converted that it is not recoverable by distillation with sulfuric acid. This is not due to the action of enzymes or to the presence of glucose. It is here shown that in determining hydrocyanic acid in plants several methods in corroboration of each other must be used.

CYANOGENESIS IN PLANTS. STUDIES ON TRIDENS FLAVUS (TALL RED TOP).[1]

By ARNO VIEHOEVER, CARL O. JOHNS, and CARL L. ALSBERG.

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(Received for publication, January 18, 1916.)

Tridens flavus (L.) Hitchcock (1) is a grass sometimes attaining a height of 6 feet. It has been described by different botanists under a variety of names. The most important synonyms are *Sieglingia sesleroides* (Mich.) Scribner, *Triodea cuprea* Jacquin, and *Poa flava* (L.). It is found widely distributed in the United States, occurring from Massachusetts to Kansas and south to Texas. The specimen described by Linnaeus was obtained from Virginia and had a yellow panicle, hence the name *flavus*. The specimen on which our work was done had a purple panicle. This form is the most common in or around the District of Columbia.

In a series of investigations on grasses (2) it was found that *Tridens flavus* yielded hydrocyanic acid. In our present work we used plants collected in August, September, and October, 1914. The hydrocyanic acid was liberated by distilling the plant with dilute sulfuric or tartaric acids and the amounts were determined colorimetrically as Prussian blue (3).

The whole plant, including the roots, collected on Aug. 18 and examined without drying, yielded 0.0075 per cent of hydrocyanic acid. In the plant collected on Sept. 22 we found only a trace of hydrocyanic acid, and none in plants collected in October. That the hydrocyanic acid was not present in the free state in the plant collected in August was shown by the fact that a distillation with steam gave a negative result.

The August plant, after drying in desiccated air at 50°C., retained 0.0066 per cent of hydrocyanic acid, and this quantity still

[1] This paper was presented at the meeting of the Society of Biological Chemists held at St. Louis in December, 1914.

remained after the dried plant had been ground and left exposed to the laboratory atmosphere for 3 months. A sample of the fresh August plant was dried in the laboratory atmosphere for more than a month. When examined at the end of this period it contained only 0.0015 per cent of hydrocyanic acid. Another sample of the same plant, stored for the same length of time at 0°C. gave 0.0013 per cent of hydrocyanic acid. Parts of the fresh August plant, dried in the same manner and for the same length of time, were examined to ascertain the distribution of the hydrocyanic acid in the plant, with the following results:

	per cent
Inflorescence tops stripped of flowers	0.0037
Stems	0.0030
Green leaves	0.0017
Dead yellow leaves	0.0000
Root	Trace.

The flowers selected for this experiment became mouldy, but after drying still gave about 0.0002 per cent of hydrocyanic acid. An analysis of flowers from the August plant, which had been stored at 0°C., was made and 0.0025 per cent of hydrocyanic acid was found. The very small amount of this acid found in the September plant was located chiefly in the unripe seeds. Fresh ripe seeds from the October plant gave a negative test.

We found that when *Tridens* was macerated for some time in water before distilling with acid, a considerable loss of hydrocyanic acid ensued. This result agrees with recent observations made by Alsberg and Black (4) on other plants and may have an important bearing on the results obtained by other workers in this line. For instance, in the investigations of Treub (5) on a large number of plants, the quantity of hydrocyanic acid obtained was increased by macerating the plant before distilling. Apparently in a number of cases where such an increase was observed after maceration, the material was distilled without the addition of a strong acid. If the macerated or unmacerated plant had been distilled together with a strong acid, different results might have been obtained, depending, of course, on the character of the cyanogenetic compound present.

Many cases are well known where hydrocyanic acid is liberated from plant material during maceration in water. The liberation

of the hydrocyanic acid in such cases is due to an enzymic reaction. For example, when bitter almonds are macerated with water, the enzyme emulsin (6), which is present together with the amygdalin, decomposes this cyanogenetic compound mainly in such a manner that the hydrocyanic acid is set free. The enzyme reaction proceeds as follows:

1. $C_6H_5.CH(CN).O.C_6H_{10}O_4.O.C_6H_{11}O_5+H_2O$
   Amygdalin
   $=C_6H_5.CH(CN).O.C_6H_{11}O_5+C_6H_{12}O_6$
   Mandelonitrile glucoside Glucose
2. $C_6H_5.CH(CN).O.C_6H_{11}O_5+H_2O=C_6H_5.CH(CN).OH+C_6H_{12}O_6$
   Mandelonitrile glucoside Mandelonitrile Glucose
3. $C_6H_5.CH(CN).OH=C_6H_5.CHO + HCN$
   Mandelonitrile Benzaldehyde Hydrocyanic acid

On the other hand, if the bitter almonds are distilled under usual conditions with acids without previous maceration the main reaction is different, resulting in the formation of mandelonitrile and glucose, only a small quantity of hydrocyanic acid being obtained.[2] The mandelonitrile is further hydrolyzed by acids to mandelic acid and ammonia. These reactions may be shown as follows:

1. $C_{20}H_{27}NO_{11}+2H_2O=C_6H_5.CH(CN).OH+2C_6H_{12}O_6$
   Amygdalin Mandelonitrile Glucose
2. $C_6H_5.CH(CN).OH+2H_2O=C_6H_5.CHOH.COOH + NH_3$
   Mandelonitrile Mandelic acid Ammonia

It has also been observed that when certain plants are macerated in dilute solutions of potassium cyanide (4) a large quantity of the potassium cyanide cannot be recovered as hydrocyanic acid by subsequent distillation with a stronger acid. *Tridens* belongs to this class of plants. When known weights of potassium cyanide were added to a mixture of the plant and water in tightly stoppered flasks which were kept at temperatures varying from 0–30°C., a loss of potassium cyanide occurred in every case and this loss increased with the period of maceration. When sodium hydroxide was added to the above mixture the loss of cyanide was

[2] Experiments on the distillation of amygdalin with acids under varying conditions have been undertaken and will be reported in another paper.

much greater. On the other hand, when tartaric acid was added to the mixture of plant, water, and potassium cyanide, there was no loss of cyanide on distilling after maceration. The loss of cyanide might be due to a chemical reaction between some of the compounds contained in the plant and the potassium cyanide. It is well known that cyanides react with a large number of organic compounds, such as aldehydes and ketones.[3]

The loss of cyanide during maceration might also be ascribed to an enzymic action, such as the action of a plant oxidase that could change the potassium cyanide to potassium cyanate.

In this connection it might be well also to consider the action of bacteria which could grow in the maceration mixture.

Adsorption of the potassium cyanide by the plant during the period of maceration might also account for this loss, since it has been found that vegetable fibers adsorb salts (7).

To ascertain if volatile aldehydes or ketones were present, which would react with the cyanide, we made an infusion of the plant with water and distilled over more than one-half of the liquid. Potassium cyanide was allowed to stand in this distillate, and on acidifying and distilling there was no loss of cyanide. A portion of the distillate was made alkaline with sodium hydroxide, potassium cyanide was added, and after standing a day all the cyanide was recovered. Hence the loss of potassium cyanide was not due to volatile aldehydes or ketones.

The plant mentioned above, which had been heated more than 1 hour during the distillation, still had the power of acting on potassium cyanide, the loss being over 20 per cent on macerating for 27 hours at 15°C. As the distillation removed only volatile products, it is still possible that the loss of potassium cyanide was due to a chemical reaction.

Attempts were also made to prevent enzyme action by boiling the plant in alcohol and by macerating with dilute alcohol. On subsequent distillation, either with or without the addition of acids, only a part or none of the available hydrocyanic acid was obtained.

[3] Dezani has shown that hydrocyanic acid is transformed to ammonia by vegetable juices (*Atti II Cong. naz. chim. appl.*, Turin, 1911). Our attention was called to this paper after we had been forced to discontinue our experiments on account of lack of material.

To exclude definitely the action of enzymes the October plant was heated in dry air up to 130°C. for 4 hours. On maceration with potassium cyanide at 15°C. for 43 hours, the loss of cyanide was about 60 per cent. The August plant, heated in the same manner, although it lost three-fourths of its available hydrocyanic acid, still acted on potassium cyanide under similar conditions, also causing a loss of about 60 per cent.

To exclude the action of bacteria and enzymes and the adsorption of potassium cyanide by plant fibers, an aqueous extract from the October plant was filtered and sterilized. Potassium cyanide was added to this liquid and a loss of 40 per cent of cyanide was observed after the mixture had stood at 15°C. for 45 hours. These results indicate that the loss of potassium cyanide is due to a chemical reaction.

That an enzyme is present in *Tridens* was shown by the action of the October plant on a solution of amygdalin. In 20 hours, 34 per cent of the amygdalin was hydrolyzed. The action of emulsin on *Tridens flavus* was tried and the same quantity of hydrocyanic acid was found as in a control experiment without emulsin.

When more material is available we intend to continue our studies on this plant and attempt to isolate the cyanogenetic compound and the enzyme.

EXPERIMENTAL PART.

The general method employed to obtain the hydrocyanic acid from the plant was as follows: The plant was placed in a flask of convenient size. The material was then covered with a measured volume of water, and sulfuric, or usually solid tartaric, acid was added to make a 5 to 10 per cent solution. The flask was immediately attached to a condenser, to the end of which a delivery tube was fitted. This tube was dipped into a dilute solution of sodium hydroxide. The mixture was boiled until the distillate no longer gave a test for hydrocyanic acid. In the case of *Tridens* all the available hydrocyanic acid was obtained before one-half of the water had distilled over. The volume of the distillate was ascertained, and an aliquot part was analyzed by converting the cyanide to Prussian blue and estimating this colorimetrically (3).

Determination of Hydrocyanic Acid in Tridens Collected on Aug. 18.

250 gm. of the whole fresh plant, including the root, were distilled without previous maceration, as described above. The yield of hydrocyanic acid was 0.0075 per cent.

A large sample of the above mentioned plant was dried at 50°C. in a stream of desiccated air. This material was then ground and left exposed to the laboratory atmosphere. Portions were analyzed at different intervals up to Nov. 9. All these analyses gave 0.0066 per cent of hydrocyanic acid.

Effect of Maceration on the Yield of Hydrocyanic Acid.

To determine the influence of maceration, experiments were made with the ground August plant dried as mentioned above. This contained 0.0066 per cent of hydrocyanic acid. The results given in Table I indicate that maceration diminished the quantity of available hydrocyanic acid.

TABLE I.

Effect of Maceration on the Yield of Hydrocyanic Acid.

Weight of plant.	Water added.	Time of maceration.	Temperature of maceration.	HCN in plant as KCN.	HCN recovered as KCN.	Loss of HCN.
gm.	*cc.*	*hrs.*	*°C.*	*gm.*	*gm.*	*per cent*
50	600	6	15	0.008	0.0057	29
50	600	18	15	0.008	0.0047	41
50	600	18	Room.	0.008	0.0040	50
100	1,000	20	"	0.016	0.0091	43
50	600	23	15	0.008	0.005	38
50	600	42	15	0.008	0.0052	35
50	600	65	15	0.008	0.0034	58
25	300	2	34	0.004	0.0036	10
25*	300	2	34	0.004	0.0036	10
25	300	24	34	0.004	0.0000	100
25*	300	24	34	0.004	0.0000	100

* 0.5 gm. of emulsin added.

Effect of Distilling with and without the Addition of Acid under Varying Conditions.

Experiments were made to determine the effect of distilling the macerated or unmacerated plant without the addition of acid. It was found that in every case distillation without the addition

of acid resulted in a partial or complete loss of the available hydrocyanic acid. Similar experiments were made in which dilute alcohol was used instead of water. The losses here were more pronounced. The results of these experiments are shown in Table II.

TABLE II.

Effect of Distilling with and without the Addition of Acid under Varying Conditions.

Weight of plant.	Water added.	Time of maceration.	Temperature of maceration.	HCN in plant as KCN	HCN recovered as KCN.	Loss of HCN.	Manner of distilling.
gm.	*cc.*	*hrs.*	*°C.*	*gm.*	*gm.*	*per cent*	
250	1,000	0	—	0.045	0.045	—	With acid.
250	1,000	0	—	0.045	None.	100	Steam without acid.
25	300 Hot water.	0	—	0.004	Trace.	Nearly 100	Without acid.
25	300	Boiled in water 30 min.	—	0.004	0.00075	81	" "
25	300	20	15	0.004	0.0036	10	With acid.
25	300	20	15	0.004	0.0030	25	Without acid.
25	300	26	15	0.004	0.003	25	" "
25	300	48	15	0.004	0.0028	30	" "
25	500 50 per cent alcohol.	0	—	0.004	None.	100	" "
25	400 50 per cent alcohol.	Boiled in 200 cc. of 95 per cent alcohol 30 min.	—	0.004	"	100	" "
25	500 50 per cent alcohol.	Boiled in the 50 per cent alcohol 15 min., macerated 39 hrs.	Room.	0.004	0.0023	42	With acid.
25	500 50 per cent alcohol.	40	"	0.004	0.001	75	Without acid.

Effect of Adding Potassium Cyanide before Maceration.

Experiments were performed to ascertain the effect of macerating the plant together with added known quantities of potassium cyanide. One series of experiments was made with the August plant which contained hydrocyanic acid (Table III). Another series of experiments was made with the October plant which did not contain hydrocyanic acid (Table IV).

TABLE III.

Effect of Adding Potassium Cyanide to the August Plant before Maceration.

Weight of plant.	Water added.	Time of maceration.	Temperature of maceration.	HCN in plant as KCN.	Weight of KCN added.	HCN recovered as KCN.	Calculated weight of KCN.	Loss of HCN.
gm.	*cc.*	*hrs.*	*°C.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
50	600	17	15	0.008	0.008	0.0107	0.016	33
6	200	168	15	0.001	0.008	0.0037	0.009	59
6	200	168	15	0.001	0.008	0.0038	0.009	58
50	600	0	—	0.008	0.008	0.014	0.016	13

TABLE IV.

Effect of Adding Potassium Cyanide to the October Plant before Maceration.

Weight of plant.	Water added.	Time of maceration.	Temperature of maceration.	HCN in plant as KCN.	Weight of KCN added.	HCN recovered as KCN.	Loss of HCN.
gm.	*cc.*	*hrs.*	*°C.*	*gm.*	*gm.*	*gm.*	*per cent*
50	500	26	15	None.	0.050	0.0107	79
100	1,000	67	15	"	0.050	None.	100
100	1,000	67	15	"	0.050	"	100
100*	1,000	23	Room.	"	0.050	0.025	50
None.**	1,000	23	"	"	0.050	0.050	None.

* In this experiment the plant was boiled in water for ½ hour, and KCN was added to this cooled mixture before macerating.

** This experiment was made as a control and conducted exactly as the previous one, except that no plant was added.

Effect of Macerating the Plant in a Solution of Tartaric Acid.

25 gm. of the August plant containing a quantity of hydrocyanic acid equivalent to 4 mg. of potassium cyanide were macerated in 300 cc. of 10 per cent tartaric acid for 48 hours at room

temperature. On distillation all the cyanide was recovered. In a check experiment 14.4 mg. of KCN were recovered instead of 15 to 16 calculated from the weight of the plant used.

Effect of Macerating the Plant in a Solution of Sodium Hydroxide.

25 gm. of plant containing a quantity of hydrocyanic acid equivalent to 4 mg. of potassium cyanide were macerated in 300 cc. of a 0.5 per cent sodium hydroxide solution for 46 hours at room temperature. Only a trace of hydrocyanic acid was recovered.

Enzymic Action of Tridens on Amygdalin.

For this experiment the October plant which contained no hydrocyanic acid was used. 50 gm. of this plant were macerated for 20 hours at room temperature in 500 cc. of water containing 0.5 gm. of Merck's amygdalin (melting point 215°C.). On distilling without adding acid, 34 per cent of the theoretical yield of hydrocyanic acid was obtained.

SUMMARY.

1. The presence of notable quantities of hydrocyanic acid in *Tridens flavus*, a common grass, has been confirmed.
2. While a considerable quantity of hydrocyanic acid was found in the plant collected in August, only a trace was found in plants collected in September, and none in October plants.
3. No free hydrocyanic acid was obtained by direct distillation with steam.
4. By maceration with water, and subsequent distillation with acid, less hydrocyanic acid was obtained than by direct distillation with acid.
5. Distillation of the macerated or unmacerated plant without the addition of acid resulted in a partial or complete loss of the available hydrocyanic acid.
6. The quantity of hydrocyanic acid obtained from *Tridens flavus* was not increased by macerating with emulsin.
7. The maximum quantity of hydrocyanic acid was found in the inflorescence tops, while only a trace was found in the roots. The ripe seeds did not contain hydrocyanic acid.

8. When the plant was macerated in water containing a known weight of potassium cyanide, considerable loss of potassium cyanide resulted. If tartaric acid was present during the maceration of the plant with potassium cyanide, all the latter could be recovered; when sodium hydroxide was present the loss of potassium cyanide was complete.

9. The loss of cyanide during maceration is probably due to a chemical reaction.

10. *Tridens flavus* contains an enzyme which hydrolyzes amygdalin.

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THE EFFECT OF SURGICAL PROCEDURES ON THE BLOOD SUGAR CONTENT.

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This paper presents the results of studies made on the blood sugar content of human beings subjected to the different influencing factors incidental to surgical procedures of various kinds. The series of studies made offers an opportunity for comparing the results obtained in human beings with those obtained on animals. In the studies made by means of animal experimentation, it has been found by various observers that a hyperglycemia occurs under many different conditions, such as fright, anesthesia, loss of blood, laparotomy, and the administration of certain drugs.

Recently one of us in cooperation with Baehr[1] found that the hyperglycemia which results from loss of blood is due to the fact that the body places into the circulation very rapidly an amount of sugar equal to that lost in the blood removed. The percentage of blood sugar rises, therefore, without any actual change in the total amount of sugar taking place in the circulating blood. A review of the literature on the subject of experimental hyperglycemia leads us to believe that in many instances the phenomenon was incidental (from the nature of the methods employed) rather than the direct result of the conditions imposed by the experiments. It is possible that with the newer and simpler methods of blood sugar analysis now at our command the conclusions arrived at will differ from those thus far recorded.

We were able, through the courtesy of the attending surgeons of the Hospital, to study the changes in the sugar content of the blood of patients requiring surgical interference. This class of cases was selected for study for the following reasons: (1) All

[1] Epstein, A. A., and Baehr, G., *J. Biol. Chem.*, 1914, xviii, 21.

such patients are obliged to fast for a period of 12 to 20 hours prior to operation. Hence we could obtain a number of determinations of the blood sugar content under similar alimentary conditions. (2) In the period of preparation, the apprehension experienced by the patient is sufficient to constitute a psychic factor. The first specimen of blood examined was obtained in the anesthesia room just prior to operation. (3) It is possible in these cases to study the effect on the sugar content of the blood, of a number of other influences; *viz.*, (a) anesthesia, general and local; (b) nature and location of the disease; (c) nature and location of the operation (whether intra- or extraperitoneal); (d) loss of blood. (4) It was also possible to study the occurrence of post-operative glycosuria and its relation to hyperglycemia.

Method.

The determination of the blood sugar content was made according to the method of Lewis and Benedict,[2] modified in certain respects for our purposes. The blood was obtained by venipuncture and three determinations were made in each case: (a) just before anesthesia; (b) directly after operation; (c) 24 hours (in some cases 48 hours) after operation.

The Blood Sugar before Anesthesia.

In all, seventy-seven cases were studied. From the determinations made prior to anesthesia (after a period of starvation varying from 12 to 20 hours) the following conclusions were made:

The sugar content of the blood in males, (ranging from 15 to 74 years in age) varied from 0.074 to 0.109 per cent. In females (ranging from 15 to 60 years in age), the readings varied from 0.066 to 0.104 per cent. Thus the average for males was 0.096 per cent, and for females 0.084 per cent, giving a general average of 0.09 per cent for both sexes (see Tables I and II).

These results correspond in general with those of other investigators. Thus Liefmann and Stern[3] using Knapp's method of analysis, found values

[2] This work was done while the micro method of blood sugar analysis devised by one of us was being perfected. Epstein, *J. Am. Med. Assn.*, 1914, lxiii, 1667.

[3] Liefmann, E., and Stern, R., *Biochem. Z.*, 1906, i, 299.

ranging from 0.065 to 0.105 per cent. They regarded 0.11 per cent as a beginning hyperglycemia. Leire, using Bang's method,[4] found values ranging from 0.06 to 0.11 per cent, in fifteen individuals of whom six had 0.11 per cent. Bang[4] found 0.10 to 0.11 per cent in three persons. Jacobsen[5] found values ranging from 0.09 to 0.116 per cent in fourteen cases, as follows: three cases had 0.09 per cent; seven had 0.10; three, 0.11; and one, 0.116. All the above readings were made in the morning before breakfast.

The age of the individuals examined seems to have no constant or definite influence upon the percentage of blood sugar. This is in agreement with the opinion of Frank.[6]

In twelve cases (Table III) in which we observed a hyperglycemia before anesthesia (a sugar content above 0.11 per cent), it was evident from further examinations that the high sugar content was due to various associated factors, and not to any psychic disturbance. These were of the nature of obesity, nephritis, hypertension, fever, and loss of blood as in menorrhagic

TABLE I.

Male Cases.

Case No.	Age.	Blood sugar.	Case No.	Age.	Blood sugar.
	years	*per cent*		*years*	*per cent*
1	22	0.102	50	29	0.087
8	29	0.095	51		0.100
10	42	0.092	56	40	0.102
13	18	0.077	57		0.090
19	40	0.085	61	58	0.093
20	49	0.085	62	30	0.109
21	43	0.074	64	51	0.099
32	16	0.077	67	15	0.097
41	49	0.085	70	26	0.102
45	17	0.090	72	26	0.089
47	50	0.095	76	33	0.099
49	30	0.091	77	74	0.100

	per cent
Highest value	0.109
Lowest "	0.074
Average	0.096

[4] Bang, I., Der Blutzucker, Wiesbaden, 1913, 30.
[5] Jacobsen, A. T. B., *Biochem. Z.*, 1913, lvi, 471.
[6] Frank, E., *Therap. Gegenwart*, 1914, lv, 439.

TABLE II.

Female Cases.

Case No.	Age.	Blood sugar.	Case No.	Age.	Blood sugar.
	years	*per cent*		*years*	*per cent*
2	38	0.088	35	17	0.089
3	35	0.108	37	36	0.080
9	37	0.086	38	50	0.091
11	18	0.070	42	61	0.104
12	22	0.078	43	35	0.103
14	21	0.087	44	25	0.082
15	15	0.070	46	19	0.076
16	22	0.092	53	26	0.084
17	21	0.097	54	44	0.103
18	42	0.103	55	21	0.078
22	20	0.067	58	26	0.077
23	33	0.078	59	31	0.080
24	38	0.066	63	19	0.090
25	30	0.095	66	26	0.084
27	30	0.078	69	50	0.090
28	28	0.069	71	25	0.092
30	40	0.063	73	46	0.107
31	38	0.083	74	46	0.109
34	42	0.107	75	36	0.095

	per cent
Highest value	0.109
Lowest "	0.063
Average	0.084

conditions, in which a hyperglycemia is believed to be encountered frequently. That the hyperglycemia in these cases was due not to psychic factors was further evidenced by the subsequent determinations of the blood of these patients, which showed that the hyperglycemia was persistent in many of them.

The influence of psychic factors, such as pain, fear of operation, and fear of anesthesia, upon the blood sugar content apparently is negligible. With the exception of the dozen cases discussed in the preceding paragraph our readings differ in no essential way from those of other determinations made under more normal environmental conditions. Our conclusion is, therefore, at variance with the results obtained in experiments on animals.

TABLE III.

Exceptional Cases.

Case No.	Age.	Sex.	Blood Sugar. A. Before anesthesia.	Blood Sugar. B. After operation.	Blood Sugar. C. 24 hours after operation.	Remarks.
	years		*per cent*	*per cent*	*per cent*	
6	19	Male.	0.146	0.176	0.120	Well nourished; febrile; not prepared (no period of starvation). Appendectomy; gas and ether 26 min.
29	19	"	0.133	0.139	0.097	Appendectomy; gas and ether 31 min.
65	49	"	0.120	0.113	0.128	Thin; nephritic; cardiac. Suprapubic cystotomy; local anesthesia 22 min.
67	30	"	0.113	0.114	0.094	Hydrocephalus; brain tumor. Trephine and corpus callosum puncture; gas and ether 32 min.
5	45	Female.	0.149	0.284	0.233	Obese; menopause 2 yrs. ago; nephritis. Cholecystectomy; cystic duct drainage; gas and ether 83 min. (urine not examined).
7	18	"	0.149	0.133	0.088	Thin; menstruating. Appendectomy (no time given).
26	36	"	0.126	0.130	0.103	Cardiac; amputation of cervix; gas and ether 43 min.
33	47	"	0.113	0.149	0.093	Obese; menopause 2 yrs. ago; albuminuria. Post-operative adhesions, gas and ether 53 min.
39	32	"	0.117	0.113	0.073	Menorrhagia. Urethroplasty; gas and ether 50 min.
40	60	"	0.118	0.232	0.200	Obese; blood pressure 180. Considerable hemorrhage during plastic operation. Gas and ether 55 min. Post-operative glycosuria shows after operation; no sugar in urine 24 hrs. later.
52	24	"	0.120	0.147		Bleeding from submucous fibroids. Curettage; gas and ether 23 min.
60	45	"	0.125	0.213	0.125	Albuminuria; obese. Cholecystectomy; gas and ether 64 min.

The Effect of Anesthesia and Operative Procedures on the Blood Sugar.

Every operation involves a number of factors which may have their separate and independent influences upon the sugar content of the blood. These factors include the nature and duration of the anesthesia, the nature and location of the operation, and the degree of shock incidental to the procedures employed. Thus it has been stated by various authors that deep narcosis can produce hyperglycemia in animals;[4] that laparotomy as contrasted with extraperitoneal operations[7] and that loss of blood[1] can cause hyperglycemia. While in studies such as we have made the effect of the individual factors cannot be isolated, it is possible by means of proper tabulation and comparison of the results in a considerable number of cases, to deduce which of the factors is chiefly responsible for the disturbance in the balance of the blood sugar. For example, if it is true that operations within the peritoneal cavity affect the blood sugar content differently from extraperitoneal operations, then we should find in intraperitoneal operations of the same duration, conducted under the same narcosis as extraperitoneal procedures, disturbances in the blood sugar content of an order different from those found in extraperitoneal operations.

Local Anesthesia (*Table IV*).—Only four cases operated upon under local anesthesia were examined, and from the results ob-

TABLE IV.

Local Anesthesia.

Case No.	Blood sugar.		Duration.	Nature of operation.
	A. Before anesthesia.	B. After operation.		
	per cent	*per cent*	*min.*	
49	0.091	0.104	6	Cauterization of rectal ulcer.
56	0.102	0.098	10	Excision of metastatic hypernephroma.
65	0.120	0.113	22	Suprapubic cystotomy.
77	0.100	0.084	15	" "
Average..	0.103	0.100		

[7] Katz, D. A., and Lichtenstern, D. R., *Biochem. Z.*, 1914, lx, 313.

tained it appears that not only was there no resultant hyperglycemia, but that a slight diminution in the blood sugar content takes place. An average of the results obtained by the authors yields these figures:

(a) 0.103 per cent (before anesthesia).
(b) 0.100 " " (after operation).

The duration of the procedure varied from 6 to 22 minutes.

Nitrous Oxide Anesthesia (Table V).—Only five cases operated upon under nitrous oxide anesthesia were examined. The average results for these were:

(a) 0.090 per cent (before anesthesia).
(b) 0.115 " " (after operation).

The duration of the procedure varied from 4 to 32 minutes, and the character of the operations was similar to that of cases subjected to nitrous oxide and ether anesthesia. The rise in percentage of blood sugar is fairly definite, but there is no regularity in the percentage increase and evidently no relation to the duration of the anesthesia.

TABLE V.

Nitrous Oxide Anesthesia.

Case No.	Blood sugar.		Duration.	Nature of operation.
	A. Before anesthesia.	B. After operation.		
	per cent	*per cent*	*min.*	
41	0.085	0.106	4	Brisement of right shoulder.
63	0.090	0.117	31	
64	0.099	0.130	32	Hydrocele.
68	0.097	0.107	25	Resetting of fractured leg.
23	0.078	0.115	13	Pelvic abscess; drainage.
Average..	0.090	0.115		

Nitrous Oxide and Ether Anesthesia (Bennett Method) (Tables VI and VII).—Sixty cases operated upon under general anesthesia administered with the Bennett inhaler (one or two bags of nitrous oxide gas with gradual shifting to pure ether) were examined. These cases may be classified and tabulated as follows: (1) lapa-

TABLE VI.

Nitrous Oxide and Ether Anesthesia.
Laparotomies.

Duration.	No. of cases.	A.	B.	C.	Increase of B over A.
min.		*per cent*	*per cent*	*per cent*	*per cent*
20–30	4	0.102	0.140	0.096	37
30–40	7	0.090	0.118	0.083	31
40–60	5	0.094	0.120	0.093	27
60–90	9	0.097	0.161	0.134	66
Over 90	2	0.089	0.143	—	61
All cases.....	27	0.096	0.138	0.109	44

TABLE VII.

Nitrous Oxide and Ether Anesthesia.
Extraperitoneal Operations.

Duration.	No. of cases.	A.	B.	C.	Increase of B over A.
Gynecological cases.					
min.		*per cent*	*per cent*	*per cent*	*per cent*
10–20	1	0.080	0.104	0.084	30
20–30	4	0.098	0.123	0.083	26
30–40	2	0.094	0.131	—	40
40–60	5	0.105	0.141	0.112	34
60–90	2	0.085	0.112	0.103	32
All cases.....	14	0.097	0.128	0.100	32
Renal cases.					
60–90	3	0.089	0.154	0.102	73
110	1	0.087	0.204	0.096	134
All cases.....	4	0.088	0.166	0.100	89
Neurological cases.					
60–90	2	0.091	0.142	0.106	56
Miscellaneous cases.					
20–30	4	0.084	0.112	0.109	33
30–40	3	0.102	0.123	0.102	21
40–60	2	0.105	0.121	0.101	15
60–90	3	0.089	0.156	0.101	76
All cases.....	12	0.093	0.127	0.104	37

rotomies; (2) extraperitoneal operations. This general grouping is made because of the reputed effect of intraperitoneal operations on the blood sugar content. In the accompanying tables the results are grouped according to the above scheme, specifying, however, the location of each group of operations and the duration of the anesthesia. Column A represents the average sugar content of the blood examined just before anesthesia; Column B represents the average sugar content directly after the operation; and Column C represents the sugar content 24 hours after operation.

In fifty-seven of the sixty cases examined there was a well marked increase in the blood sugar content after anesthesia and operation. In one case (No. 9, a plastic lasting 70 minutes) there was a rise of only 0.001 per cent within the limit of error. In another case (No. 39, Table III, a urethroplasty lasting 50 minutes) there was a decrease of 0.004 per cent, in a third a decrease of 0.006 per cent.

The average results in all kinds of cases, regardless of duration, showed a decided increase after operation, and the persistence of a moderate increase 24 hours later. Reference to Tables VI, VII, VIII, and IX will give the figures for the different groups of cases studied.

TABLE VIII.

Nitrous Oxide and Ether Anesthesia.
Summary, According to Nature of Operation.

Nature of operation.	No. of cases.	A.	B.	C.	Increase of B over A.
		per cent	*per cent*	*per cent*	*per cent*
Intraperitoneal	27	0.096	0.138	0.109	44
Gynecological	14	0.097	0.128	0.100	32
Renal	4	0.088	0.166	0.100	89
Neurological	2	0.091	0.142	0.106	56
Miscellaneous including	12	0.093	0.127	0.104	37
Hernias	4	0.099	0.138	0.106	40
Thyroids (not exophthalmic)	2	0.093	0.133	0.110	43

In cases (Table IX) which were under anesthesia for less than 1 hour the average percentage increase of B over A varied from 15 to 37 per cent. The increase of only 15 per cent was seen in two

TABLE IX.

Nitrous Oxide and Ether Anesthesia.
Laparotomies and Non-laparotomies, Grouped According to Duration of Anesthesia.

	No. of cases.	A.	B.	C.	Increase of B over A.
		per cent	*per cent*	*per cent*	*per cent*
Duration 20–30 min.					
Laparotomies	4	0.102	0.140	0.096	37
Non-laparotomies	8	0.091	0.118	0.096	30
Duration 30–40 min.					
Laparotomies	7	0.090	0.118	0.083	31
Non-laparotomies	5	0.098	0.127	0.102	30
Duration 40–60 min.					
Laparotomies	5	0.094	0.120	0.093	27
Non-laparotomies	7	0.105	0.135	0.109	30
Duration 60–90 min.					
Laparotomies	9	0.097	0.161	0.134	66
Non-laparotomies	10	0.089	0.144	0.103	62
Of these,					
Renal cases	5	0.090	0.149	0.103	65
Others	5	0.087	0.138	0.102	59

miscellaneous extraperitoneal procedures lasting 40 to 60 minutes. The increase of 37 per cent was seen in a group of four laparotomies lasting 20 to 30 minutes.

In cases (Table IX) which were under anesthesia for more than 1 hour the average percentage increase varied from 32 to 89 per cent. The increase of 32 per cent was seen in two gynecological cases, plastic operations lasting 60 to 90 minutes. The increase of 89 per cent was seen in a group of four nephrectomies lasting from 60 to 110 minutes.

The greatest percentage increase of B over A was seen in this group of nephrectomies (Table VIII). In Case 50 (nephrectomy for hydronephrosis, lasting 110 minutes) the readings were A, 0.087, B, 0.204, C, 0.096 per cent, a rise of 134 per cent of B over A, the largest increase in the series of cases examined.

From a study of Fig. 1 we conclude that: (a) There was no difference between the effect of intraperitoneal operations and extraperitoneal operations upon the sugar content of the blood. (b) The anesthesia played the important rôle in the production

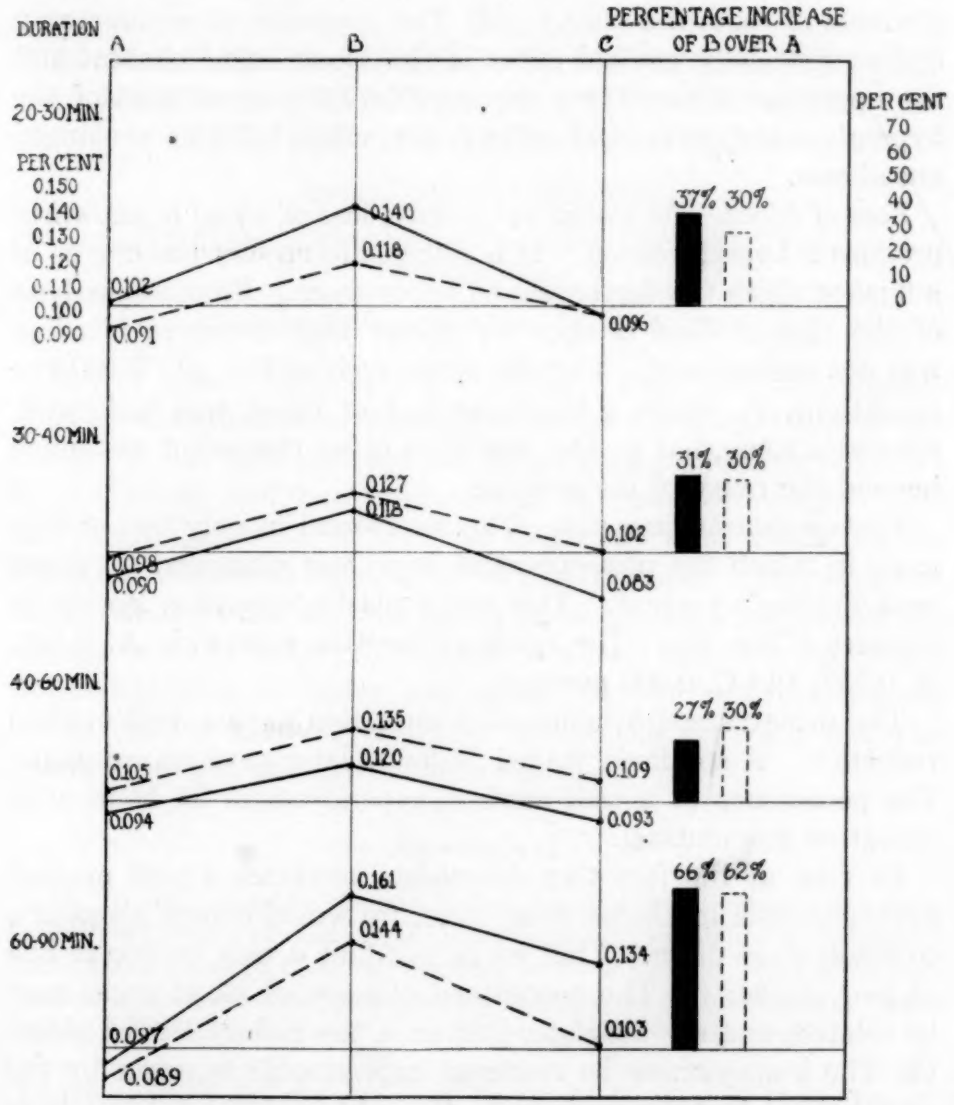

FIG. 1. Chart showing the course of the glycemia (Table IX). A, before; B, at end of anesthesia; C, 24 hours later. Vertical lines, percentage increase of sugar after anesthesia of various duration. Continuous lines, laparotomies. Broken lines, extraperitoneal operations.

of the hyperglycemia observed. (c) The more marked increases in blood sugar content appeared when the anesthesia lasted 1 hour or more. (Bang stated that deep narcosis produced hyperglycemia of its own accord.) (d) The operation of nephrectomy had an especially marked effect in the blood sugar content and the operation of itself was responsible for a good part of the hyperglycemia, over and above the effect of the prolonged anesthesia.

Loss of Blood.—As stated previously, loss of blood is known to produce a hyperglycemia. It is difficult to specify the degree of influence which this factor played in our cases. From an analysis of the clinical data it may be stated that excessive bleeding was not encountered. Certain cases, such as No. 42 (Wertheim operation), in which a moderate loss of blood was incidental, showed a somewhat greater rise than other cases, but not much beyond the range of the average.

Post-operative Glycosuria.—This was found in only one of fifty cases in which the post-operative urine was examined for sugar with Fehling's reagent. This was a plastic operation lasting 55 minutes (Case 40). The readings for this case were A, 0.118, B, 0.232, and C, 0.200 per cent.

The urine passed 8 hours after operation gave a well marked reduction. A specimen voided 24 hours later gave no reduction. The persistence of a well marked hyperglycemia 24 hours after operation was unusual.

In view of the fact that anesthesia produces a well marked hyperglycemia in almost every case, we would expect glycosuria to result more often. That we do not find it may be due to one of two causes: (1) The percentage increase of blood sugar may be relative and not absolute, just as in the case of loss of blood. (2) The kidneys may be rendered impermeable to sugar by the anesthetic. Further work upon this phase of the subject is in progress.

THE FORMATION OF SPECIFIC PROTEOCLASTIC FERMENTS IN RESPONSE TO THE PARENTERAL INJECTION OF FOREIGN PROTEINS.*

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The so called Abderhalden reaction, dating from the appearance of Abderhalden's *Schutzfermente des tierischen Organismus*, has given rise to a vast amount of work on proteolytic ferments in the blood, and has produced an extensive literature. The fact that the reaction would be of diagnostic value, if practicable, has given additional impetus to the investigations. Few workers, except Abderhalden and his pupils, have obtained results definite enough to be of value. This lack of results has been attributed by Abderhalden to faulty technique.[1] It was to avoid the use of the dialysis method[2] and thus escape this criticism that the technique in the present instance was used.

HISTORICAL.

Ide,[3] in 1902, came to the conclusion that "it was impossible to form antibodies in the body against the most important substance of bacteria," nucleoproteins; while Galeotti,[4] working in the same year, reports the production of an antiserum to anthrax by injecting its nucleoproteins. MacCallum,[5] Portis,[6] and Yates[7] stated that antithyroid serum produced by

* Dissertation for the degree of Ph.D., University of Pennsylvania, 1916.

[1] Abderhalden, E., *Münch. med. Woch.*, 1914, lxi, 233.

[2] Abderhalden, Abwehrfermente, Berlin, 3rd edition, 1913.

[3] Ide, M., *Fortschr. Med.*, 1902, xx, 833.

[4] Galeotti, G., *Primo Cong. patol. ital.*, Turin, 1902, cited in *Biochem. Centr.*, 1903, i, 276.

[5] MacCallum, W. G., *Med. News*, 1903, lxxxiii, 820.

[6] Portis, M. M., *J. Infect. Dis.*, 1904, i, 127.

[7] Yates, J. L., *Univ. Penn. Med. Bull.*, 1903–04, xvi, 195.

them had no specific action, while Bierry[8] stated that the action of such antiserum, while not specific, was more pronounced on the organ from which the nucleoprotein was derived. Levene[9] concluded from his results on hemolytic sera produced by different constituents of erythrocytes that nucleoproteins may be effective in forming antibodies. Pearce[10] in his early and in his most recent work has shown that specific cytotoxic sera cannot be produced by the injection of nucleoproteins. All these workers attacked the problems rather from the viewpoint of the immunologist than from that of the chemist.

Hedin[11] first showed that the blood of the ox contained a weak proteolytic enzyme that acted in alkaline medium, but which was prevented from acting in the blood by the presence of antibodies. Delezenne and Pozerski[12] found that the proteolytic powers were increased when the blood serum was incubated over chloroform. In 1904, Kawasoye[13] applied his results to the diagnosis of pregnancy and reported that he obtained a placental antiserum by sensitization of the animal to placenta or albuminous urine from pregnant women. Wells and Osborne[14] have shown that guinea pigs become immune to the chief vegetable proteins of their food, while on the other hand, Besredka[15] could not succeed in sensitizing a guinea pig to milk either by oral or rectal administration. Abderhalden[16] found on the injection of nucleoproteins and nucleins that a reinjection in no case caused death, inferring therefrom the production of protective ferments. Schawlow[17] reported the existence of specific proteolytic ferments in the blood in specific pathologic conditions; he has used the Abderhalden test to diagnose carcinoma and sarcoma and reported failure in only 7 and 6 per cent.

Voelkel[18] has been able to secure a ninhydrin reacting substance with antisera to diphtheria, typhoid and anthrax bacilli, trypanosomes, and spirochetes. Schwarz,[19] using the Abderhalden technique, reported the

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[8] Bierry, H., *Compt. rend. Soc. biol.*, 1903, lv, 476.

[9] Levene, P. A., *J. Med. Research*, 1904, xii, 191.

[10] Pearce, R. M., and Jackson, H. C., *J. Infect. Dis.*, 1906, iii, 742. Pearce, R. M., *Studies from Bender Hyg. Lab.*, 1904, i. Pearce, R. M., Karsner, H. T., and Eisenbrey, A. B., *J. Exp. Med.*, 1911, xiv, 44. Williams, P. F., and Pearce, R. M., *Surg. Gyn. and Obst.*, 1913, xvi, 411.

[11] Hedin, S. G., *J. Physiol.*, 1904, xxx, 195.

[12] Delezenne, C., and Pozerski, E., *Compt. rend. Soc. biol.*, 1903, lv, 327.

[13] Kawasoye, Ueber die biochemische Diagnose der Schwangerschaft, Inaug. Diss., Erlangen, 1904.

[14] Wells, H. G., and Osborne, T. B., *J. Infect. Dis.*, 1911, viii, 77.

[15] Besredka, *Ann. l'Inst. Pasteur*, 1909, xxiii, 166.

[16] Abderhalden, E., and Kashiwado, T., *Z. physiol. Chem.*, 1912, lxxxi, 285.

[17] Schawlow, A., *Münch. med. Woch.*, 1914, lxi, 1386.

[18] Voelkel, E., *Münch. med. Woch.*, 1914, lxi, 349.

[19] Schwarz, H., *J. Am. Med. Assn.*, 1914, lxiii, 371.

positive diagnosis of cancer and pregnancy. Echols[20] states that the dialysis method is of value when negative, showing the absence of pregnancy, but that a positive result may be due to errors in technique. Carlson,[21] in answer to the last two statements, says that the Abderhalden test is not qualitative but quantitative, and that one could probably get a positive test in 100 per cent of the cases if the time for digestion were extended, as polyvalent ferments are present always in the blood. This latter position is supported by many investigators. Flatow[22] finds that the splitting of placenta occurs not only in the pregnant serum, but also that the proteolytic ferments of the normal serum break up the proteins of the placenta unspecifically and quantitatively. He[23] also objects to Abderhalden's results on the basis of his using "cooked" placenta, saying that "if specific ferments were present they would not show with cooked organs." In his later work[24] with casein, he finds that casein is digested by the normal serum and more intensely with the pregnant serum. Michaelis and Lagermarck[25] were not able to demonstrate a specific ferment for placenta, but obtained positive results even with men and old women in certain pathologic conditions. The results of many investigators indicate the presence of proteolytic ferments which evidently increase in quantity under certain conditions.

Herzfeld[26] found the percentage of amino-acid greater in pregnant than in normal serum, indicating that the pregnant serum had increased fermentative powers. Schlimpert and Issel[27] report increased ferments in animals during pregnancy, but they were not specific for the animals' own placenta. According to the recent investigations of Jobling, Eggstein, and Petersen,[28] placental tissue is not digested but actually resists, in an increased degree, enzyme action. Kolmer and Williams[29] are of the opinion that during pregnancy there is an increase of the general proteolytic ferment of the serum; and recently they[30] state that there are two sets of ferments in pregnant serum; (1) normal non-specific ferment, and (2) specific; the former released through the adsorption of the antiferment by non-specific substances, the second released only through specific protein antigen.

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[20] Echols, C. M., *J. Am. Med. Assn.*, 1914, lxiii, 370.

[21] Carlson, A. J., *J. Am. Med. Assn.*, 1914, lxiii, 1176.

[22] Flatow, L., *Münch. med. Woch.*, 1914, lxi, 468.

[23] Flatow, *Münch. med. Woch.*, 1914, lxi, 1168.

[24] Flatow, *Münch. med. Woch.*, 1914, lxi, 1500.

[25] Michaelis, L., and Lagermarck, L. v., *Deutsch. med. Woch.*, 1914, xl, 316.

[26] Herzfeld, E., *Biochem. Z.*, 1914, lix, 249.

[27] Schlimpert, H., and Issel, E., *Münch. med. Woch.*, 1913, lx, 1758.

[28] Jobling, J. W., Eggstein, A. A., and Petersen, W., *J. Exp. Med.*, 1915, xxi, 239.

[29] Kolmer, J. A., and Williams, P. F., *Am. J. Obst.*, 1915, lxxi, 899.

[30] Kolmer and Williams, *Am. J. Obst.*, 1915, lxxii, 1.

The immunologists have also offered explanations for this increased activity during pregnancy and after injection of protein, as an immunity reaction. Vaughan[31] states that he procures in guinea pigs, by immunization, a serum which will digest egg protein. He explains immunity as development of a serum which will digest foreign bodies and render them harmless. Jobling and Petersen[32] explain this digestion in blood as due to non-specific proteolytic ferments or proteases normally present in blood and held in check by antiferments, which they believe to be unsaturated fatty acids. Bronfenbrenner[33] explains the reaction by saying that the results obtained by Abderhalden are due to the presence in this serum of specific substances that are not fermentative in nature, and that the substratum is not digested, but that autolysis takes place and serum proteins are liberated, thus giving rise to protein digestion products in the blood. On the other hand, we have the evidence of Frank[34] that no experimental specific immune reaction to placenta can be demonstrated. Lake[35] also regards the possibility of producing an immune serum of therapeutic value in chorion epithelioma, by use of human placenta, as extremely slight. Abderhalden[36] regards the diagnosis of pregnancy and carcinoma as dependent on the presence of ferments produced by the injection of the protein or tissue in question. De Waele,[37] however, did not succeed in preparing a serum that would give digestion with specific tissues, but he did find that the addition of even an inorganic substance will cause the liberation of dialyzable, ninhydrin-reacting substances. DeWaele,[38] and Heilner and Petri[39] show that ferments appear very quickly after parenteral injection of the protein, in intervals hardly sufficient for the elaboration of new and specific ferments; they support the theory that the ferments are preformed and that the substratum serves to activate rather than to bring about the production of new ferments.

The most recent work done on proteoclastic ferments tends to favor the opinion that the production of ferments during pregnancy and after the injection of protein is a quantitative and not a qualitative reaction. Sloan[40] observed that there may be an increase of proteolytic activity un-

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[31] Vaughan, V. C., *J. Am. Med. Assn.*, 1914, lxiii, 365.

[32] Jobling, J. W., and Petersen, W., *J. Exp. Med.*, 1914, xix, 459; *Bull. Johns Hopkins Hosp.*, 1915, xxvi, 356.

[33] Bronfenbrenner, J., Schlesinger, M. J., and Mitchell, W. T., *J. Am. Med. Assn.*, 1915, lxv, 1268.

[34] Frank, R. T., *J. Exp. Med.*, 1907, ix, 263.

[35] Lake, G. C., *J. Infect. Dis.*, 1914, xiv, 385.

[36] Abderhalden, *Fermentforschung*, 1914, i, 20.

[37] De Waele, H., *Z. Immunitätsforsch., Orig.*, 1914, xxii, 170.

[38] De Waele, *Z. Immunitätsforsch., Orig.*, 1909–10, iv, 148.

[39] Heilner, E., and Petri, T., *Münch. med. Woch.*, 1913, lx, 1530.

[40] Sloan, LeR. H., *Am. J. Physiol.*, 1915, xxxix, 9.

der the above conditions. Van Slyke, Vinograd-Villchur, and Losee[41] found nearly or quite as much power to digest placenta in normal as in pregnant sera. Malone[42] reported that he has found ferments, even in the urine, which would digest placenta. The clinical literature on the subject, supporting the efficiency of the test and also denying its usefulness, would fill volumes and has been omitted here, because we are concerned with the reaction from the chemical rather than from the clinical viewpoint. A full list of references may be found in Abderhalden's *Abwehrfermente*.

EXPERIMENTAL.

As stated in the introduction, the object of this investigation was to determine whether proteoclastic ferments or activity develop in the blood in response to the injection of a foreign protein and cause digestion when allowed to act on the protein injected, using a method which would not be open to the criticism of the dialysis method or of the ninhydrin reaction. The method followed in each case was practically the same. White rabbits were injected with an amount of protein varying from 100 to 800 mg. in solution or suspension, depending on its solubility in Ringer's solution. The Ringer's solution was boiled and cooled to about 40°C. The protein was placed in this and after the hair had been cut from the rabbit and the skin disinfected with iodine, this solution was injected parenterally. Each rabbit received three successive injections, 1 week apart. The day following the last injection the rabbit was etherized and bled to death from the carotid. The blood was caught in a beaker and was stirred continuously to defibrinate it. The control animals were bled in the same way. The defibrinated blood was centrifuged, the serum decanted off, and portions were used to obtain the figures for the non-protein nitrogen according to the method of Folin,[43] except that we used, for some of the later tests, absolute ethyl alcohol instead of methyl alcohol.

The rest of the serum was divided into 5 or 10 cc. portions ac-

[41] Van Slyke, D. D., and Vinograd, M., *Proc. Soc. Exp. Biol. and Med.*, 1914, xi, 154. Van Slyke, D. D., Vinograd-Villchur, M., and Losee, J. R., *J. Biol. Chem.*, 1915, xxiii, 377.

[42] Malone, R. H., *Proc. Soc. Exp. Biol. and Med.*, 1915, xii, 126.

[43] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 527.

cording to the amount obtained, and these were diluted up to 50 cc. with Ringer's solution, made alkaline with 2 cc. 0.1 N NaOH and placed in a flask with 1 gm. of the particular protein, and incubated at 37.5°C. The first three were incubated for 24 hours, the next two for 48 hours, and the last two for 42 hours. After incubation the contents of the flask were mixed with ten volumes of alcohol, and allowed to stand for 24 hours. The filtrate was treated with a saturated alcoholic solution of $ZnCl_2$, allowed to stand 24 hours, and a Kjeldahl run on the filtrate. Bearing in mind the fact that Greenwald[44] could not recover all the amino-acid from an alcoholic filtrate, we did some check analyses with 2.5 per cent trichloroacetic acid, as recommended by him, but since the results checked so closely with the alcohol figures, we continued with the alcohol method.

The results are summarized in the tables.

TABLE I.

Experiment.	Animal.	Injection.			Non-protein N in 100 cc.	
		1.	2.	3.	Before digestion.	After digestion.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*
Protamine	Experimental	100	100	100	.....	0.039
	"	100	100	100	.....	0.042
	Normal	...	...	...	.....	0.048
Casein	Experimental	500	500	500	0.040	0.041
	Normal	...	...	...	0.021	0.037
Bence-Jones	Experimental	250	125	250	0.034	0.059
	Normal	...	...	...	0.037	0.060
Phaseolin	Experimental	800	500	500	0.036	0.039
	Normal	...	...	...	0.039	0.035
Edestin	Experimental	500	450	400	0.039	0.045
	Normal	...	...	...	0.036	0.041
Gliadin	Experimental	350	450	400	0.035	0.039
	Normal	...	...	...	0.100	0.100
Soy bean globulin	Experimental	250	250	250	0.064	0.062
	Normal	...	...	...	0.036	0.047
Milk albumin	Experimental	250	250	250	0.037	0.052
	Normal	...	...	...	0.036	0.048

[44] Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.

TABLE II.

Digestion with	Increase of non-protein N.	
	Injected animals.	Normal animals.
	mg.	*mg.*
Protamine	..	..
Casein	1	16
Bence-Jones	25	23
Phaseolin	3	−4
Edestin	6	5
Gliadin	4	0
Soy bean globulin	−2	11
Milk albumin	15	12

DISCUSSION.

The results show plainly in all cases that under the conditions of the experiment there is practically no digestion with the blood of the injected animal in excess of that which takes place with the serum of the normal control animal. The greatest amount of digestion takes place in the case of the Bence-Jones protein and milk albumin, which would naturally be expected, as these two proteins are more nearly related to mammalian proteins. With casein, the blood of the injected animal on the addition of casein gave a precipitin reaction, so that this might account for the greater increase of nitrogen in the normal animal; the precipitin reaction might preclude the possibility of a proteolytic action taking place. The results would also lead one to suppose that the blood of the rabbit possesses to a slight degree proteolytic activity, but that this activity toward a particular protein is not increased by injecting the animal with that protein. The criticism might be offered that these proteins are too foreign; but if the reaction must be discriminated on the basis of the protein being just foreign enough, it would seem that the possibility of usefulness becomes remote.

The very premise that ferments (free or restrained) exist preformed in the blood is not strongly borne out in these data. The ferments are best regarded as endocellular entities; and under certain conditions, not well understood, the cellular activity may be so stimulated that an excess of ferment is produced and overflows

into the blood stream (just as frequently trypsin occurs in the urine), but exists there not primarily for the purpose of digestion directly in the circulating blood. The fact that such varying results on the presence or activity of ferments in the blood have been obtained might be due to the fact that some of the investigators have accidentally reproduced the conditions under which the cells are stimulated to greater activity and the excess ferments then appeared in the blood. Our results would lead us to believe that pure proteins at least need not call forth such increased activity. Even though there be in pregnancy a specific serum reaction, it is probably not one due to placental digestion in the blood.

It is not demonstrated that the normal hydrolysis of the protein of the body occurs in the circulating blood; indeed, it is much more likely that this occurs largely within the cells of the tissues. It is easily possible to imagine introduced foreign protein being taken into the body cells and hydrolyzed there, without any reaction appearing in the circulating plasma. It may be shown that foreign protein introduced into the venous blood may be taken up by the tissues.[45] In like manner, it might be imagined that the placental cells entering the maternal circulation would be held and hydrolyzed within the tissues, and leave no trace of any enzymic or other activity in the circulating plasma. In theory, therefore, one must separate the question of hydrolysis of foreign proteins from the question of the reaction of the maternal body to placental cells. It is entirely possible that there is a specific reaction of the host to placental cells or to neoplasms, without this being a hydrolysis of their proteins in the circulating blood dependent on the presence there of enzymic activity.

SUMMARY.

Protamine, phaseolin, and gliadin are not digested to any degree by either normal serum or that of an animal injected with these substances.

Casein and soy bean globulin are digested to a greater extent by the normal serum than by that of the injected animal.

[45] Unpublished data.

Edestin and milk albumin are digested to the same degree by the normal and experimental serum.

Bence-Jones protein is digested to a marked degree by both sera and equally well in each case.

I take this opportunity to thank Professor Alonzo E. Taylor, at whose suggestion this work was undertaken and under whose direction it was carried out, for his help, both in the experimental work and in reading and revising the manuscript. I also thank Professor Mendel and Dr. Osborne for the proteins[46] supplied by them, Dr. Isaac F. Harris,[47] for those supplied by him, and Drs. Taylor and Miller for the Bence-Jones protein.

[46] Casein, edestin, gliadin, phaseolin.

[47] Soy bean globulin, milk albumin.

THE LIMIT OF ASSIMILATION OF GLUCOSE.

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By common consent, rather than by accurate experimentation, the limit of assimilation of glucose on alimentary administration has been set at 200 to 250 gm. on the empty stomach. From this figure downwards, the student of diabetes applies the test; from this figure upwards the student of the diseases of the ductless glands applies the test. The tests herein reported were made upon healthy medical students. The tests were made as follows: Breakfast of a roll or one slice of toast and a cup of tea or coffee was allowed. 2½ to 3 hours afterwards, the glucose was administered in strong solution, the sugar of the blood having been estimated. 3 hours after the administration of the glucose, the blood was again subjected to estimation of glucose. The method used was that of Bang, in triplicate, the blank determined each day. The urine was collected over 24 hours. Normal urine frequently contains a faint trace of sugar. The test we applied was essentially Benedict's procedure, the solution being allowed to stand after heating for 2 hours. Any excess of glucose above the normal trace will clearly be revealed in the test applied in this manner. The dosage of glucose (which was a pure glucose prepared from the grape) was 200 gm. in the first series, 300 gm. in the second, and 400 gm. in the last. The subjects in the second series were students who had displayed no glucosuria in the first test; the subjects of the third series were men who had displayed no reaction in the second series. The results of the estimations of sugar in the blood before and 3 hours after ingestion of the stated dose of glucose, and the results of tests for sugar in the urine subsequently secreted, are given in the table.

Sugar Data.

Blood sugar content at		Urine.	Blood sugar content at		Urine.
10 a. m.	1 p. m.		10 a. m.	1 p. m.	
200 gm. glucose.			200 gm. glucose.		
per cent	*per cent*		*per cent*	*per cent*	
0.09	0.09		*0.08	0.10	
0.10	0.10		0.10	0.09	
0.12	0.08		0.11	–	
0.11	0.11		300 gm. glucose.		
0.12	0.12				
0.11	0.15	Slightly +	*0.10	0.16	
0.12	0.12		0.14	0.16	Slightly +
0.08	0.07	Slightly +	0.11	0.13	+
0.09	0.08	++	*0.07	0.14	
*0.08	0.13		0.13	0.14	
*0.09	0.12		0.11	0.13	+
0.08	0.09	+	0.10	0.10	
0.09	0.10		0.07	0.09	
0.07	0.09	+	*0.10	0.14	–
0.08	0.09		400 gm. glucose.		
*0.07	0.10	+			
0.10	0.09				
0.10	0.07		0.14	0.12	–
0.10	0.12		0.07	0.07	–
0.08	0.10		*0.10	0.13	+
*0.06	0.11		0.07	0.09	Slightly +
0.10	0.07		0.10	0.09	–
*0.12	0.15		0.09	0.09	–

* The asterisks indicate cases where there was a rise in blood sugar greater than might be due to the experimental error in the method.

From the figures it is clear that nearly all the subjects tolerated the ingestion of 200 gm. without exhibition of glucosuria. Of nine subjects who ingested 300 gm. only three displayed glucosuria. Of the six who ingested 400 gm. only two had glucosuria. The figures for the blood sugars run within the limits to be observed with the use of the Bang method. In some instances the figure is at the very upper limit of the normal, but these figures were by no means observed following the largest ingestions. The figures for blood sugar are of relative value only, because of the time fixed for the estimations, 3 hours after ingestion. It was to be expected that this arbitrary time was too early for the top of

the wave in some subjects, too late in others. The general conclusion may be drawn, however, that such ingestions do not markedly influence the sugar content of the blood.

Two points in the technique required checking. Were results of tests done 2½ to 3 hours after a light breakfast as described, assumed to hold for the empty stomach? To determine this point, 400 gm. of glucose were given four times, without blood examinations, in each instance with negative results. Did the presence of lemon juice, sometimes given to flavor the large mass of syrup, modify the result? 400 gm. were given four times, without lemon juice, on the empty stomach, with negative results. Are 400 gm. the limit of ingestion? In five instances, without blood examinations, 500 gm. were given, with the production of glucosuria in but one. We regard 500 gm. as the physical limit of ingestion, except in one who has trained to the test; it is very large in bulk, inclines to nauseate, and apparently the excess is not rapidly absorbed, so that the test probably means no more than does the administration of 400 gm., which is usually tolerated.

Polyuria occurred rarely, and there was no relationship between the polyuria and glucosuria. Intestinal disturbances were not observed. An attempt was made to repeat the series with levulose, beginning at 300 gm. on account of scarcity of the pure material. But we found that, as a rule, our healthy students could not ingest 300 gm. of levulose without intestinal disturbances. 400 gm. administered to three men caused profuse diarrhea with copious watery stools, without sugar in the sparsely secreted urine. Whether this result is inherent in such amounts of levulose or whether due to some impurity in the supposedly pure preparation we used, could not be determined.

Apparently there is in the majority of healthy adult males no limit of assimilation of glucose; glucosuria does not occur following the largest possible ingestions of pure glucose. These results confirm the findings of Woodyatt, Sansum, and Wilder.[1]

[1] Woodyatt, R. T., Sansum, W. D., and Wilder, R. M., *J. Am. Med. Assn.*, 1915, lxv, 2067.